

Tissue Engineering for Conjunctival Reconstruction

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Declaration

I, Stefan Schrader confirm that the work presented in this thesis is my own.

Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Reconstruction of the conjunctiva is an essential part of ocular surface regeneration, especially if an extensive area or the whole ocular surface is affected, such as in patients with ocular cicatricial pemphigoid, Stevens-Johnson syndrome or chemical/thermal burns. However, there is a lack of suitable donor tissue for conjunctival replacement, especially when large grafts are required and it is important that new materials and methods are developed for conjunctival reconstruction. The aims of this thesis were; to characterise the conjunctival epithelial cell population and to improve the maintenance of the epithelial progenitor cells during *in vitro* expansion in order to produce conjunctival epithelial cells suitable for therapeutic use. The final aim was to transfer these cells to compressed collagen matrices and amniotic membrane and test the properties of these cell-matrix constructs. Experiments showed that cryopreservation does not alter the proliferative potential of conjunctival epithelial progenitor cells. It was also demonstrated that the maintenance of conjunctival epithelial progenitor cells during cell expansion can be improved by mimicking an environment *in vitro*, which is more similar to the stem cell niche *in vivo* and that this is accompanied by downregulation of key genes in the wnt signaling pathway. The final experimental series showed that after *in vitro* expansion, conjunctival epithelial cells can be successfully transferred and cultured on amniotic membrane and compressed collagen gels. In conclusion these studies highlighted the complexity of tissue engineering ocular surface substitutes and provided further clues for the goal to obtain a stable conjunctival substitute, suitable for transplantation.

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List of Abbreviations

| | |
|--------|--|
| ABS | Adult Bovine Serum |
| AM | Amniotic membrane |
| CCND1 | Cyclin D1 |
| CECM | Conjunctival Epithelial Culture Medium |
| CK | Cytokeratin |
| CTNNB1 | β -Catenin |
| DAPI | 4'-6-Diamidino-2-phenylindole |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl Sulfoxide |
| EGF | Epidermal Growth Factor |
| FBS | Fetal Bovine Serum |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| HCEC | Human Conjunctival Epithelial Cells |
| HCF | Human Conjunctival Fibroblasts |
| H & E | haematoxylin and eosin |
| MMC | Mitomycin C |
| MUC | Mucin |
| PAS | periodic acid-Schiff |
| PBS | Phosphate-Buffered Saline |
| PC | Plastic compressed |
| PCR | Polymerase Chain Reaction |
| PFA | Paraformaldehyde |

| | |
|--------|---|
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| TA | Transient Amplifying |
| TFF | Trefoil factor family |

Table of Contents

| | |
|---|-----------|
| 1. Introduction | 17 |
| 1.1. The conjunctiva | 17 |
| 1.1.1. Tear film..... | 17 |
| 1.1.2. Conjunctival epithelium and stroma..... | 18 |
| 1.1.3. Conjunctival epithelial stem cells..... | 20 |
| 1.1.3.1. Definitions of stem cells | 20 |
| 1.1.3.2. Stem cells of the ocular surface..... | 21 |
| 1.1.3.3. Putative epithelial stem cell markers..... | 23 |
| 1.1.4. Pattern of cytokeratin expression in ocular surface epithelia | 25 |
| 1.2. Conjunctival reconstruction..... | 27 |
| 1.2.1. Pathologies that require conjunctival reconstruction | 27 |
| 1.2.2. Techniques used for ocular surface reconstruction..... | 28 |
| 1.2.2.1. Conjunctival autografts | 29 |
| 1.2.2.2. Oral mucous membrane grafts | 29 |
| 1.2.2.3. Autologous nasal turbinate mucosa grafts..... | 30 |
| 1.2.2.4. Posterior lamella reconstruction..... | 31 |
| 1.3. Tissue engineering for conjunctival substitutes..... | 32 |
| 1.3.1. Principles of tissue engineering for ocular surface reconstruction | 32 |
| 1.3.2. Acellular polymers for matrix substitution | 35 |
| 1.3.3. Collagen matrix with an epithelial layer | 37 |
| 1.3.3.1. Extracellular matrix | 37 |
| 1.3.3.2. Fibroblast co-culture and air-lifting..... | 38 |
| 1.3.4. Simple amniotic membrane transplantation | 39 |
| 1.3.5. Amniotic membrane based cell sheets..... | 41 |
| 1.3.5.1. AM carrier for expanded conjunctival epithelial cells | 41 |
| 1.3.5.2. Oral mucosal epithelial cell sheets..... | 43 |
| 1.4. Conclusions and aims | 44 |
| 2. Materials and Methods..... | 49 |
| 2.1. Cell culture | 49 |
| 2.1.1. Conjunctival research tissue..... | 49 |
| 2.1.2. Isolation and culture of conjunctival epithelial cells | 49 |
| 2.1.3. Maintenance of 3T3 mouse fibroblasts for use as a feeder layer..... | 50 |
| 2.1.4. Growth arrest of 3T3 fibroblasts | 50 |
| 2.1.5. Visualisation of cell morphology using light microscopy..... | 51 |

| | | |
|-------------|--|-----------|
| 2.1.6. | Cell counting with a haemocytometer..... | 51 |
| 2.1.7. | Cryopreservation and thawing protocol for epithelial cells | 52 |
| 2.1.8. | Cell viability assessment | 53 |
| 2.2. | General methods and assays..... | 53 |
| 2.2.1. | Colony forming efficiency assay | 53 |
| 2.2.2. | Clonal analysis | 54 |
| 2.2.3. | Immunocytochemistry..... | 55 |
| 2.2.3.1. | Cell fixation | 55 |
| 2.2.3.2. | Imunostaining protocol..... | 56 |
| 2.2.4. | Immunohistochemistry..... | 56 |
| 2.2.5. | Histological staining of paraffin sections..... | 57 |
| 2.2.6. | Semi-quantitative RT-PCR | 58 |
| 2.2.7. | Western blot analysis | 59 |
| 2.2.8. | Calculation of cell population doublings | 60 |
| 2.2.9. | Statistical Analysis..... | 61 |
| 3. | Characterisation and expansion of the conjunctival epithelial cell population <i>in vitro</i>..... | 63 |
| 3.1. | Introduction | 63 |
| 3.2. | Methods..... | 64 |
| 3.3. | Results | 64 |
| 3.3.1. | Characterisation of the human conjunctival biopsies | 64 |
| 3.3.2. | Cell culture model for conjunctival epithelial cells | 66 |
| 3.3.3. | Characterisation of the conjunctival epithelial cell population <i>in vitro</i> | 67 |
| 3.3.4. | Colony forming capacity and clonal analysis..... | 69 |
| 3.4. | Discussion | 71 |
| 4. | Evaluation of the effect of long-term <i>in vitro</i> culture and cryopreservation on conjunctival epithelial progenitor cells..... | 74 |
| 4.1. | Introduction | 74 |
| 4.2. | Methods..... | 75 |
| 4.2.1. | Experimental setup for the cryopreservation experiments | 75 |
| 4.3. | Results | 76 |
| 4.3.1. | Morphology of conjunctival epithelial cells during long-term <i>in vitro</i> expansion | 76 |
| 4.3.2. | Immunohistochemistry and semi-quantitative RT-PCR..... | 79 |

| | |
|--|------------|
| 4.3.3. Cell viability and colony forming efficiency | 84 |
| 4.3.4. Colony forming efficiency and cell doubling rate during serial cultivation 85 | |
| 4.4. Discussion | 88 |
| 5. Simulation of an environment <i>in vitro</i> which is more similar to the stem cell niche <i>in vivo</i> in order to preserve conjunctival epithelial progenitor cells during cell expansion <i>in vitro</i> | 94 |
| 5.1. Introduction | 94 |
| 5.2. Methods..... | 97 |
| 5.2.1. Human conjunctival fibroblast Isolation and culture | 97 |
| 5.2.2. Setup of the conjunctival epithelial cell/conjunctival fibroblast co-cultures 97 | |
| 5.2.3. Two step trypsinisation method | 98 |
| 5.2.4. Comparison of the cell proliferation dynamics in the HCEC-HCF co- culture system under serum free and serum containing conditions | 101 |
| 5.3. Results | 102 |
| 5.3.1. Proliferation of conjunctival epithelial cells in the HCEC-HCF system using different epithelial cell : fibroblast ratios | 102 |
| 5.3.2. Cell growth and morphology | 104 |
| 5.3.3. Cell Proliferation in the HCEC-HCF Co-culture System | 106 |
| 5.3.4. Expression of Progenitor Cell Markers | 107 |
| 5.3.5. Goblet Cell Differentiation..... | 110 |
| 5.3.6. Colony Forming Efficiency | 112 |
| 5.4. Discussion | 114 |
| 6. Wnt signaling in an <i>in vitro</i> niche model for conjunctival progenitor cells | 120 |
| 6.1. Introduction | 120 |
| 6.2. Methods..... | 121 |
| 6.2.1. Analysis of gene expression by Affymetrix GeneChip® human genome arrays 121 | |
| 6.2.1.1. The principles of microarray technology | 121 |
| 6.2.1.2. Affymetrix oligonucleotide arrays | 122 |
| 6.2.1.3. Sample processing and steps of the expression array | 123 |
| 6.2.1.3.1 RNA isolation and quality control..... | 123 |
| 6.2.1.3.2 Hybridization and array scanning | 124 |

| | | |
|-------------|---|------------|
| 6.2.1.3.3 | Microarray data analysis..... | 125 |
| 6.2.1.3.4 | Gene ontology (GO) and pathway profile analysis..... | 126 |
| 6.2.2. | Reverse transcription (RT) and quantitative real-time PCR | 126 |
| 6.3. | Results | 129 |
| 6.3.1. | Affymetrix microarray analysis..... | 129 |
| 6.3.1.1. | RNA quality control results..... | 129 |
| 6.3.1.2. | Microarray quality control results | 131 |
| 6.3.1.3. | Microarray gene expression analysis of HCEC cultured under HCEC-HCF and HCEC-3T3 conditions | 139 |
| 6.3.2. | Analysis of differences in Wnt signaling associated gene expression between HCEC-HCF and HCEC-3T3 conditions by quantitative PCR | 142 |
| 6.3.2.1. | Protein Expression of β -Catenin and Cyclin D1 (CCND1)..... | 146 |
| 6.4. | Discussion | 148 |
| 7. | Exploration of amniotic membrane and compressed collagen gels as a carrier for <i>in vitro</i> expanded conjunctival epithelial cells..... | 153 |
| 7.1. | Introduction | 153 |
| 7.2. | Methods..... | 154 |
| 7.2.1. | Preparation of Human Amniotic Membrane | 155 |
| 7.2.2. | Preparation of the plastic compressed collagen gel matrices | 156 |
| 7.2.3. | Break strength tests..... | 158 |
| 7.3. | Results | 160 |
| 7.3.1. | Denuding of the amniotic membrane..... | 160 |
| 7.3.2. | Evaluation of different conjunctival epithelial cell densities on PC collagen gels and AM | 161 |
| 7.3.2.1. | Comparison of PC collagen gels with and without fibroblasts and AM as carriers for conjunctival epithelial cells | 164 |
| 7.3.2.2. | Break strength tests | 166 |
| 7.4. | Discussion | 168 |
| 8. | General Discussion..... | 171 |
| 8.1. | Conclusion | 179 |
| 9. | Future Work | 182 |
| 9.1. | Improvement of the culture conditions for conjunctival epithelial cells in order to maintain the stem cell population during cell expansion <i>in vitro</i> | 182 |
| 9.2. | Evaluation of PC collagen gels as a substrate for the maintenance of conjunctival epithelial progenitor cells | 183 |

| | |
|---|-----|
| 9.3. Mechanical evaluation of PC collagen gels as a conjunctival substitute | 184 |
| 9.4. Steps towards a clinical application..... | 185 |
| 10. References | 189 |
| 11. Publications arising from this thesis | 215 |
| 11.1. Original articles | 215 |
| 11.2. Reviews | 215 |

List of Figures

| | |
|--|----|
| Figure 1: Composition of the Ocular Surface: Conjunctiva (Red), Cornea (Blue), Limbus..... | 17 |
| Figure 2: Conjunctival tissue section, showing the different components of the conjunctiva: non-keratinized stratified epithelium, basement membrane and a well vascularized stroma..... | 19 |
| Figure 3: Distribution of stem cells on the ocular surface. | 23 |
| Figure 4: Ocular surfaces from patients suffering from ocular cicatricial pemphigoid. | 28 |
| Figure 5: Haemocytometer grid for cell counting. | 52 |
| Figure 6: Conjunctival biopsy location and characterization..... | 65 |
| Figure 7: Cell culture model for conjunctival epithelial cells. | 67 |
| Figure 8: Conjunctival epithelial cells characterization. | 68 |
| Figure 9: Colony forming efficiency and clonal analysis from conjunctival epithelial cells grown out of explants culture. | 70 |
| Figure 10: Experimental sequence for the cryopreservation experiments..... | 76 |

| | |
|---|-----|
| Figure 11: Morphology of non-cryopreserved (B-E) and for 14 days cryopreserved conjunctival epithelial cells (F-I) during serial cultivation. . | 79 |
| Figure 12: Expression of cytokeratins and putative progenitor cell-like markers at P ₁ as assessed by immunostaining..... | 80 |
| Figure 13: Putative progenitor cell marker expression during serial expansion at P ₁ (A, E), P ₂ (B, F), P ₃ (C, G) and P ₄ (D, H) for p63 α (A-D) and ABCG2 (E-H). | 82 |
| Figure 14: Results of RT-PCR analysis from P ₁ -P ₄ | 84 |
| Figure 15: Cell viability and colony forming efficiency. | 85 |
| Figure 16: Colony forming efficiency and cumulative cell doubling rate from non-cryopreserved conjunctival epithelial cells (P ₀ -P ₃) and conjunctival epithelial cells cryopreserved for 14 days (P ₀ F-P ₃ F). | 87 |
| Figure 17: Interactions in the stem cell niche. | 96 |
| Figure 18: Experimental setup of the HCEC-HCF co-cultures and the HCEC-3T3 control group..... | 98 |
| Figure 19: Two step trypsinisation method. | 100 |
| Figure 20: Proliferation of conjunctival epithelial cells in the HCEC-HCF co-culture system at day 1, 3, 6, using different seeding ratios of epithelial cells and fibroblasts. | 103 |
| Figure 21: Cell growth under HCEC-HCF (A-C) and HCEC-3T3 (D-F) conditions..... | 105 |
| Figure 22: Expression of Ki67 in the HCEC-HCF co-culture system under serum free conditions and in the presence of serum..... | 107 |

| | |
|---|-----|
| Figure 23: Expression of putative progenitor cell markers under HCEC-HCF (A-C) and HCEC-3T3 (D-F) conditions. | 108 |
| Figure 24: Conjunctival epithelial cell colonies grown in the presence of serum using cells transferred from the HCEC-HCF (A-C) and HCEC-3T3 (D-F) co-culture systems. | 110 |
| Figure 25: Expression of the goblet cell specific mucin MUC5AC and PAS-staining under HCEC-3T3 and HCEC-HCF conditions. | 112 |
| Figure 26: Comparison of the total colony forming efficiency and colonies with a surface area > 10mm ² between cells cultured under HCEC-HCF and HCEC-3T3 conditions. | 113 |
| Figure 27: Steps of the Affymetrix GeneChip whole transcript sense target labeling assay (image adapted from affymetrix.com) | 125 |
| Figure 28: RNA quality control by the Agilent Bioanalyzer for the samples from the HCEC-HCF and HCEC-3T3 (control) group. | 130 |
| Figure 29: Quality control of the microarray experiments. | 134 |
| Figure 30: Quality control of the microarray experiments. | 136 |
| Figure 31: Quality control of the microarray experiments. | 138 |
| Figure 32: Plot of the positive and negative control together with an area under the curve (AUC) plot. | 138 |
| Figure 33: Microarray gene expression analysis of HCEC cultured under HCEC-HCF and HCEC-3T3 conditions. | 141 |
| Figure 34: Scheme of the Wnt signaling pathway. | 144 |
| Figure 35: Up- and downregulation of genes with a significant difference in gene expression ($p \leq 0.05$) and a fold change (FC) ≥ 1.5 | 145 |

| | |
|---|-----|
| Figure 36: Protein Expression of β -Catenin and Cyclin D1 (CCND1) under HCEC-HCF and HCEC-3T3 conditions. | 147 |
| Figure 37: Preparation of the amniotic membrane | 156 |
| Figure 38: Process of plastic compression. | 158 |
| Figure 39: Break strength testing device. | 160 |
| Figure 40: Denudation procedure of the AM..... | 161 |
| Figure 41: Evaluation of different seeding densities of epithelial cells on PC collagen gels after one week of submerged culture. Scale bars, 100 μ m. | 163 |
| Figure 42: Evaluation of different seeding densities of epithelial cells on AM after one week of submerged culture. Scale bars, 100 μ m. | 164 |
| Figure 43: Comparison of PC collagen gels with and without fibroblasts and AM as carriers for conjunctival epithelial cells. Scale bars, 100 μ m. | 165 |
| Figure 44: Break strength tests of pc collagen gels, AM and human conjunctiva..... | 167 |

List of Tables

| | |
|--|-----|
| Table 1: Primary antibodies for immunostaining and westernblot. | 57 |
| Table 2: Primer sequences for semi-quantitative RT-PCR..... | 59 |
| Table 3: Genes table of interrogated genes by the SABiosciences human Wnt signaling PCR-array (PAHS-043) | 129 |
| Table 4: File- and sample names for the HCEC-HCF and the HCEC-3T3 (control) group. | 131 |

Table 5: Analysis of differences in Wnt signaling associated gene expression
between HCEC-HCF and HCEC-3T3 conditions by quantitative PCR...143

Chapter 1:

Introduction

1. Introduction

1.1. The conjunctiva

The conjunctiva is a transparent mucous membrane formed of a stratified epithelium and a well-vascularised stroma. Its surface can be divided into the bulbar, forniceal and palpebral regions, which together with the cornea, the limbus and the tear film constitute the ocular surface (Fig. 1). The main functions of the conjunctiva are secretion of the mucin component of the tear film, formation of a mechanical barrier against pathogens and contribution to the immune defence of the ocular surface.

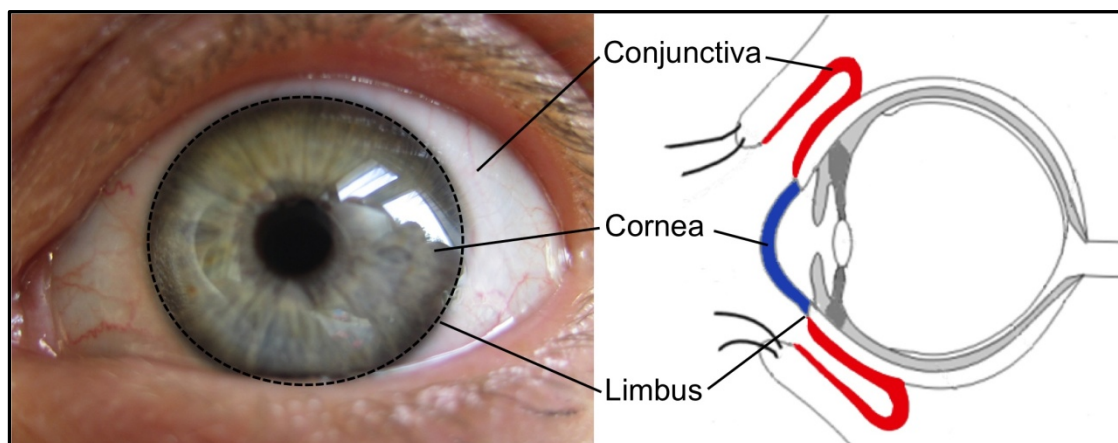


Figure 1: Composition of the Ocular Surface: Conjunctiva (Red), Cornea (Blue), Limbus

1.1.1. Tear film

The precorneal tear film contains water, protective antimicrobial proteins, cytokines, lipids and mucins. Historically it has often been subdivided into a

lipid, aqueous and mucinous layers. The lipid component is secreted by the meibomian glands in the eyelid and forms the superficial layer of the tear film. The aqueous component contains electrolytes, water and a variety of proteins, peptides and glycopeptides, which are primarily secreted by the lacrimal gland and the accessory lacrimal glands of the lids. The mucous component is mainly produced by conjunctival goblet cells, however corneal epithelial cells and lacrimal gland cells have also been shown to produce mucins (Paulsen 2008).

1.1.2. Conjunctival epithelium and stroma

The conjunctival epithelium is a non-keratinized, stratified layer. It consists of a population of goblet and non-goblet epithelial cells (Fig. 2). The conjunctival and corneal epithelium together provide a specialized interface that is important for tear film stability. The apical membranes at the surface of the epithelial cells form undulating ridges, termed microplicae, that increase the surface area. Emanating from the apical surface is a mucous layer called the glycocalyx, which contains transmembrane bound mucins (MUC 1, 4 and 16) derived from the epithelial cells themselves (Paulsen 2008). Bound to the transmembrane mucins are soluble mucins (MUC5AC) and the trefoil factor family (TFF) peptides TFF1 and TFF3 secreted by the conjunctival goblet cells. The mucins and the TFF peptides together form a gel that determines the non-Newtonian rheological properties of the tearfilm, promote migration of corneal cells, have anti-apoptotic properties and induce cell scattering (Langer, Jagla et

al. 1999; Paulsen 2008). Conjunctival and corneal epithelial cells also produce antimicrobial peptides (e.g. defensins) that can react against pathogens (McDermott 2004).

The conjunctival stroma consists of strong vascularised connective tissue that is separated from the epithelium by a basement membrane. Beside its function as a mechanically stable and elastic matrix for the conjunctival epithelium, it contains organized lymphoid tissue (conjunctiva-associated lymphoid tissue – CALT), which together with the lacrimal gland and the efferent tear duct system take part in the antimicrobial defence of the ocular surface (Knop and Knop 2003; Paulsen 2008).

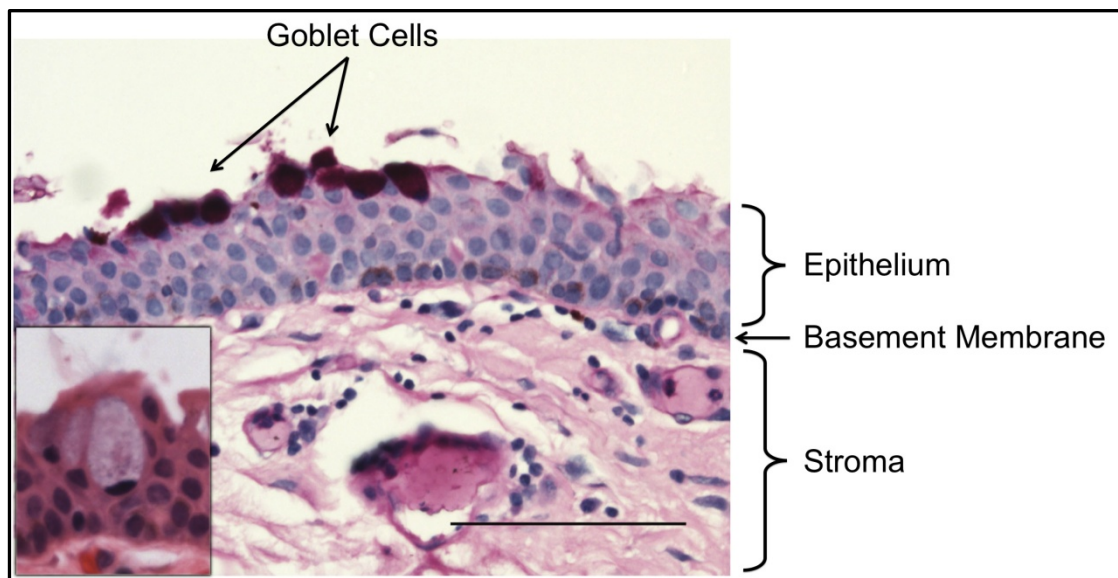


Figure 2: Conjunctival tissue section, showing the different components of the conjunctiva: non-keratinized stratified epithelium, basement membrane and a well vascularized stroma.

The epithelium consists of non-goblet and goblet epithelium cells. PAS-Staining, scale bar 100 μ m. Insert: Magnification of a goblet cell with its typical goblet shape (HE-Staining). The cell shows polarity with a basal nucleus and apical secretory mucins.

1.1.3. Conjunctival epithelial stem cells

1.1.3.1. Definitions of stem cells

The classic definition of a stem cell requires, that it posses two qualities, first: self-renewal - the ability to go through numerous cycles of cell division while maintaining the undifferentiated state and second: potency - the capacity to differentiate into specialized cell types (Alison, Poulsom et al. 2002; Bongso and Richards 2004).

There are two main types of stem cells, embryonic and adult. Embryonic stem cells are derived from isolated inner cell masses of mammalian blastocysts. A blastocyst is an embryo in the early stage of development, more specifically an embryo that is about 6 days old and consists of about 120 cells. After removal from the blastocyst the cells can be cultured *in vitro* in order to establish an embryonic stem cell line. The cell line remains pluripotent and can theoretically be induced to differentiate into any specialised cell type of choice.

However, adult stem cells (also known as somatic stem cells) are thought to be present in all tissues and responsible for maintaining their specific tissue. Adult stem cells are found in extremely small numbers in the body and are difficult to isolate. Only 1 in 10^7 - 10^8 cells are thought to be adult stem cells (Matikainen and Laine 2005).

However, there is great potential benefit in studying adult stem cells. One of the major advantages of using adult, rather than embryonic stem cells is that these cells can be harvested from the patient, expanded *in vitro* and reintroduced into the same patient, thereby avoiding the risk of rejection and

the need for immunosuppression likely to be required with the use of embryonic stem cells. Also, there are fewer ethical issues associated with the use of adult rather than embryonic stem cells for research and possible clinical treatments and finally the use of embryonic stem cells for transplantation bears the risk of teratoma induction in the host (Bongso and Richards 2004).

1.1.3.2. Stem cells of the ocular surface

The ocular surface is covered by two stratified constantly renewing tissues, the limbal-corneal and the conjunctival epithelia. These closely related but distinct cell lineages (Kruse, Chen et al. 1990; Wei, Wu et al. 1993) arise simultaneously from a few Pax6-positive ectodermal cells that remain on the embryonic ectodermal surface following formation of the lens vesicle (Davis and Reed 1996; Koroma, Yang et al. 1997; Wolosin, Schutte et al. 2002). The conjunctival epithelium has a rapid cell-turnover, and its stem cells are believed to be present within the tissue, supplying differentiated conjunctival epithelial cells throughout the lifetime of the host (Lavker and Sun 2003).

The distribution of ocular surface stem cells has been widely debated. In the traditional concept corneal epithelial stem cells are situated in the limbus region (Cotsarelis, Cheng et al. 1989) although recent findings in the mouse suggest that they might be distributed throughout the corneal surface (Majo, Rochat et al. 2008). In the conjunctiva, stem cell numbers were thought to be enriched in the fornix region (Wei, Cotsarelis et al. 1995). However, the limbus (Pe'er, Zajicek et al. 1996), bulbar conjunctiva (Pellegrini, Golisano et al. 1999), palpebral conjunctiva (Chen, Ishikawa et al. 2003) and mucocutaneous

junction (Wirtschafter, Ketcham et al. 1999) have also been proposed as stem cell rich locations. In a recent study by Nagasaki et al. the movement of conjunctival epithelial cells in mice was monitored in vivo using GFP labelled cells. The mitotic rate of the GFP positive cells was also examined and the distribution of label retaining cells (LRCs) analysed. They showed that bulbar conjunctival epithelial cells were mitotically active indicating that they were capable of self-renewal in-situ, that the LRCs were distributed uniformly in the bulbar conjunctiva, and that no significant lateral cell migration occurred (Nagasaki and Zhao 2005). These conclusions are in agreement with the findings of Pellegrini et al. who demonstrated a uniform distribution of cells with high proliferative capacity, which would include stem cells, in both the bulbar conjunctiva and fornix. Their results also indicated that conjunctival non-goblet, and mucin-producing goblet cells are derived from a common bipotent progenitor and that differentiation into goblet cells occurs relatively late, such that goblet cells may be generated from transient amplifying cells (Pellegrini, Golisano et al. 1999) (Fig. 3).

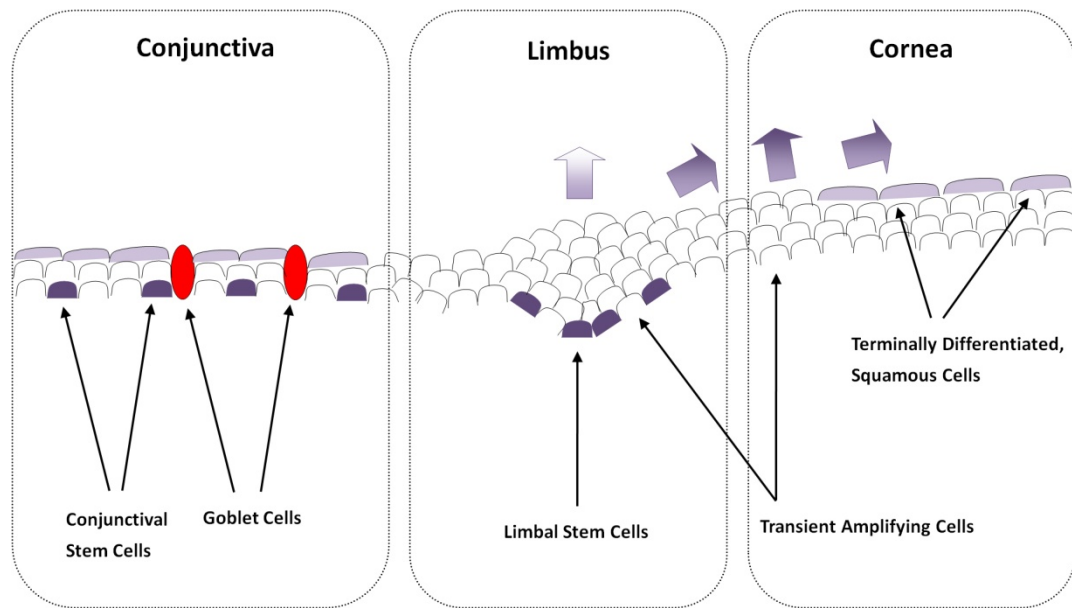


Figure 3: Distribution of stem cells on the ocular surface.

Limbal stem cells are enriched in the limbus region, give rise to transient amplifying cells, which migrate towards the central cornea and eventually become terminally differentiated and squamous. Conjunctival stem cells however seem to be distributed uniformly in the basal layer of the conjunctiva and give rise to goblet and non-goblet epithelial cells.

1.1.3.3. Putative epithelial stem cell markers

A number of putative markers for epithelial stem cells have been proposed (Zieske, Bukusoglu et al. 1992; Jones and Watt 1993; Li, Simmons et al. 1998), however a specific stem cell marker remains elusive. Initially, the identification of stem cells relied on their high proliferative capacity *in vitro* (Barrandon and Green 1987; Rochat, Kobayashi et al. 1994; Mathor, Ferrari et al. 1996; Dellambra, Vailly et al. 1998) and the slow cycling of [³H]TdR- and BrdU by these cells *in vivo* (Cotsarelis, Cheng et al. 1989; Cotsarelis, Sun et al. 1990; Lavker, Miller et al. 1993; Lehrer, Sun et al. 1998). More recently the

p63 transcription factor has shown to be expressed by epidermal and limbal holoclones but to be undetectable in paraclones, and it therefore might distinguish epithelial stem cells from transient amplifying cells (Pellegrini, Dellambra et al. 2001). It has also been found that among the six existing p63 isoforms, the $\Delta Np63\alpha$ isoform is the most dominant form in epithelial cells with stem cell like characteristics (Kawasaki, Tanioka et al. 2006).

ABCG2, a membrane transporter protein has also been suggested as a putative marker of epithelial stem cells (Watanabe, Nishida et al. 2004). In a study by Budak et al. the efflux of the dye “Hoechst 33342”, which is mediated by the ABCG2 transporter, was compared in limbal-corneal and conjunctival epithelial cells. Flow cytometry showed a side population (SP) of <1% of cells that expressed ABCG2. This SP contained a high percentage of cells that showed slow cycling prior to tissue collection, exhibited an initial delay in proliferation after culturing and displayed clonogenic capacity, and were resistant to phorbol-induced differentiation. Immunohistochemistry showed ABCG2 expression in clusters of conjunctival and limbal basal epithelial cells but not in the central corneal epithelium. The authors concluded that the SP phenotype might be useful for the selection of conjunctival and limbal basal cell populations that are enriched in stem cells (Budak, Alpdogan et al. 2005).

Another marker that has been recently proposed as a potential stem cell marker for epithelial cells is cytokeratin 15, as it has shown to be expressed by hair follicle progenitor cells (Lyle, Christofidou-Solomidou et al. 1998) and limbal epithelial cells with progenitor cell characteristics (Blazejewska, Schlotzer-Schrehardt et al. 2009). Other markers, which were used to identify

ocular surface progenitor cells include integrin $\beta 1$ (Notara, Schrader et al. 2011), C/EBP δ (Barbaro, Testa et al. 2007) and K14 (Zhao, Allinson et al. 2008). However, none of these markers can be considered as specific. Therefore, to identify epithelial stem cells a combination of various cell markers, the epithelial cell morphology and functional tests of the proliferative potential of the cells have to be combined to evaluate a progenitor cell population.

1.1.4. Pattern of cytokeratin expression in ocular surface epithelia

The pattern of cytokeratin expression can be used to verify the phenotype of ocular surface cells as well as tissue-engineered ocular surface substitutes. It is crucial that epithelial cells cultivated *in vitro* are characterised before transplantation to ensure a good quality graft. Cytokeratins belong to the intermediate sized filaments, which together with the microfilaments and the microtubules form the cytoskeleton of the eukaryotic cell. There are two subfamilies of cytokeratins, the acidic type I and the neutral/basic type II. Only heterodimers of type I and type II cytokeratins can form intermediate filaments, and cytokeratin filaments are therefore always heteropolymers formed from equal numbers of type I and type II cytokeratin subunits. A single epithelial cell can make a variety of cytokeratins, all of which copolymerize into a single keratin filament system. The cytokeratin pattern varies among different epithelial cell types and therefore a given epithelium or epithelial cell can be characterized by its specific pattern of cytokeratin expression (Moll, Franke et al. 1982; Kurpakus, Maniaci et al. 1994).

The CK3/12 pair is considered to be a cornea-type differentiation marker (Meller and Tseng 1999). However, CK3 has been shown to be expressed by conjunctival cells *in vivo* and *in vitro* (Yen, Pflugfelder et al. 1992; Chen, Mui et al. 1994; Diebold, Calonge et al. 1997), whereas CK12 seems to be more specific for genuine corneal terminal differentiation (Chen, Mui et al. 1994). CK19 is expressed in numerous simple and stratified squamous epithelia, including those of the cornea and conjunctiva. It is uniformly expressed in all layers of the conjunctiva *in vivo* and *in vitro* (Moll, Franke et al. 1982; Risse Marsh, Massaro-Giordano et al. 2002). The cornea exhibits a more irregular mosaic-like CK19 staining pattern in the peripheral corneal zone *in vivo* (Moll, Franke et al. 1982; Risse Marsh, Massaro-Giordano et al. 2002) and there are also variations in staining intensity *in vitro* (Risse Marsh, Massaro-Giordano et al. 2002). The CK4/13 pair is thought to be specific for non-keratinized stratified epithelia. CK4 is expressed in the conjunctiva and cornea *in vivo*. In the conjunctiva CK4 is found in all epithelial layers, but expression in the basal levels seems to be weaker and more focal. In the cornea only the superficial epithelial layers seems to express CK4 *in vivo* (Kurpakus, Maniaci et al. 1994; Meller and Tseng 1999; Risse Marsh, Massaro-Giordano et al. 2002). Conjunctival and corneal cells *in vitro* have shown immunoreactivity to CK4 in the suprabasal and superficial cells, but with almost no staining of the basal cells.

1.2. Conjunctival reconstruction

1.2.1. Pathologies that require conjunctival reconstruction

Many ocular surface disorders such as ocular cicatricial pemphigoid, Stevens-Johnson syndrome, toxic epidermal necrolysis and chemical/thermal burns can lead to severe conjunctival damage and scarring. This can result in fornix shortening, symblepharon and finally ankyloblepharon (Fig. 4). Cicatrisation of the fornices, especially the inferior fornix, causes entropion and trichiasis, which can persistently damage the ocular surface epithelia and result in recurrent erosions, ulcer formation and secondary bacterial infection (Ormerod, Fong et al. 1988). Also, disruption of the tear film and dry eye disease can occur due to ineffective blinking, reduction of the tear meniscus, loss of goblet cells and keratinisation of the ocular surface epithelia (Ralph 1975; Solomon, Espana et al. 2003). The resultant prolonged inflammation causes depletion of the limbal epithelial stem cell population that leads to corneal conjunctivalisation and neovascularisation, ingrowth of subepithelial fibrous tissue, and stromal scarring. Ultimately there can be severe ocular discomfort and blindness from corneal opacity (Shapiro, Friend et al. 1981; Dua and Forrester 1990) (Fig. 4B).

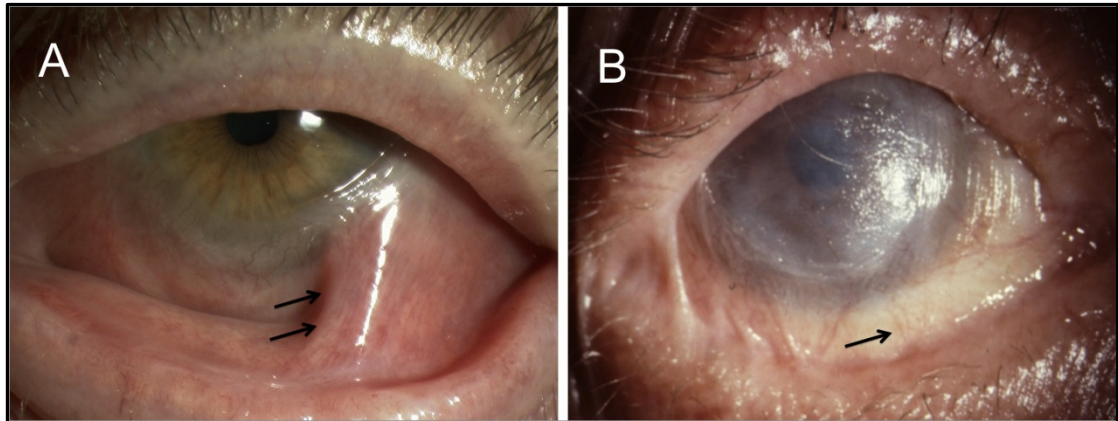


Figure 4: Ocular surfaces from patients suffering from ocular cicatricial pemphigoid.

A: symblepharon formation (arrows) and B: progressed disease presenting fornix shortening, cicatrised inferior fornix (arrow), ankyloblepharon and corneal opacity.

1.2.2. Techniques used for ocular surface reconstruction

In recent years several techniques such as keratolimbal transplantation, amniotic membrane (AM) grafting, and delivery of *in vitro* cultured epithelial stem cell sheets derived from the corneal limbus or alternative sources such as oral mucosa, have been developed to restore the corneal surface (Kenyon and Tseng 1989; Tsai and Tseng 1994; Tseng, Prabhasawat et al. 1998; Nakamura, Inatomi et al. 2004). However, before corneal reconstruction is performed it is very important that the ocular surface environment is optimised and a deep fornix is established because success largely depends on normal conjunctival function (Barabino, Rolando et al. 2003; Espana, Di Pascuale et al. 2004; Tseng, Di Pascuale et al. 2005). Several surgical approaches to reconstruct the conjunctival fornix have been described. They all involve excision of the scar tissue and application of a tissue substitute.

The ideal conjunctival substitute should consist of a stable, thin and elastic matrix that is well tolerated. In addition, the matrix should carry a suitable layer of donor epithelium with a self renewal potential, as there is a diffuse loss of conjunctival epithelial cells and goblet cells in severe ocular surface diseases. The tissue substitutes that have been successfully used for conjunctival reconstruction are conjunctival autografts, oral mucous membrane, nasal turbinate mucosa and AM (Hosni 1974; Thoft 1977; Vastine, Stewart et al. 1982; Shore, Foster et al. 1992; Kuckelkorn, Schrage et al. 1996; Solomon, Espana et al. 2003).

1.2.2.1. Conjunctival autografts

Conjunctival autografting has been used successfully to cover small conjunctival defects, e.g. following pterygium excision (Thoft 1977; Vastine, Stewart et al. 1982). However, the size of conjunctival autografts available is limited and would certainly be insufficient for the reconstruction of an entire fornix in a fellow eye. Also in autoimmune mediated inflammatory conditions, such as Stevens-Johnson syndrome or ocular cicatricial pemphigoid, any trauma to the conjunctiva could reactivate the underlying process and therefore should be avoided.

1.2.2.2. Oral mucous membrane grafts

Grafts of oral mucous membrane have been widely used for fornix reconstruction (Hosni 1974; Shore, Foster et al. 1992). Oral mucosa is easily available, and although complications are uncommon, donor site morbidity can

limit the availability of tissue if large full-thickness grafts are required. In addition, oral mucin deficiency may be present in severe cicatricial pemphigoid or Stevens-Johnson syndrome, which limits the usefulness of oral mucosa for conjunctival reconstruction in these conditions. Other limitations include the risk of postoperative shrinkage and cicatrization of the membrane (Karesh and Putterman 1988), as well as the cosmetically apparent difference in bulk, colour and texture of the tissue.

1.2.2.3. Autologous nasal turbinate mucosa grafts

The nasal mucosa is capable of providing substitute mucin as it not only contains subepithelial mucin glands (such as are present also in the oral mucosa) but also a large number of goblet cells in the surface epithelium. Furthermore, it is only rarely affected in systemic mucosal inflammatory disorders such as cicatricial pemphigoid. The first successful use of nasal mucosal grafts for fornix reconstruction was reported by Kuckelkorn et al. who successfully reconstructed the fornices in 13 of 17 patients with ocular burns (Kuckelkorn, Schrage et al. 1996). In another study Wenkel et al. reported the use of nasal mucosa grafts on 55 eyes (50 patients) who had severe fornix shortening following ocular burns or systemic cicatricial mucosal disease (Wenkel, Rummelt et al. 2000). In this report mucin production increased after surgery and the persistence of goblet cells on the ocular surface could be shown for up to 10 years. There was also a reduction of symptoms in 94% of the patients, and in 42% the visual acuity improved. The disadvantage of nasal mucosal grafts is that, while sufficient amounts can be harvested, the

extraction of the tissue is complex as it requires partial resection of the inferior or middle nasal turbinate, removal of all bone or cavernous tissue, and thinning of the mucosa. Furthermore none of these procedures are without morbidity to the donor site.

1.2.2.4. Posterior lamella reconstruction

Clinical anatomy divides the eyelid into an anterior (skin and orbicularis oculis muscle) and posterior (tarsus and conjunctiva) lamella. Full-thickness eyelid defects that exceed 25% of the eyelid width cannot be closed directly and therefore require a full thickness graft (Spinelli and Jelks 1993). For posterior lamella reconstruction the graft needs to consist of a plate of dense fibrous tissue as a tarsus equivalent and a mucous membrane surface similar to the conjunctiva. Tarsconjunctival grafts are ideal as they represent the original tissue, but an adequate graft size is often difficult to obtain. When native tissue is insufficient, a variety of mucosal grafts may be considered. Hard palate grafts are often utilized because they possess a smooth, moist non-keratinized surface, provide good structural support and may be relatively easily harvested (Rafii and Enepekides 2006). In a study by Ito et al. hard palate mucoperiosteal grafts were used for posterior lamellar reconstruction and the authors reported that the hard palate mucosa was excellent for reconstruction of the conjunctival surface, whereas the periosteum acted as a supporting tissue (Ito, Suzuki et al. 2001; Ito, Fujiwara et al. 2007). Auricular (Baylis, Rosen et al. 1982; Hashikawa, Tahara et al. 2005) or nasoseptal (Millard 1962) cartilage grafts have also been used, but these grafts can be too thick

and stiff to achieve appropriate contact between the eyelid and eyeball, resulting in an unnaturally firm eyelid (Baylis, Rosen et al. 1982; Bartley and Kay 1989; Cohen and Shorr 1992; Ito, Fujiwara et al. 2007). In a recent study by Barbera et al. (Barbera, Manzoni et al. 2008) venous wall grafts have been introduced for posterior lamellar reconstruction in seven patients with invasive carcinomas of the eye lid. The patients were treated with a full wall superficial vein graft to replace the tarsal conjunctival layer. The reconstructed eyelids had an appropriate thickness, a deep fornix and good cosmetic results. No complication or graft failure was observed in any patient during an average follow-up of 4.6 years. According to the authors this new approach might be a useful alternative method which is easy to perform and gives good functional and aesthetic results.

1.3. Tissue engineering for conjunctival substitutes

1.3.1. Principles of tissue engineering for ocular surface reconstruction

A suitable ocular surface substitute must consist of a stable and elastic matrix that can be integrated into the ocular surface tissue without causing inflammation, and this scaffold must be covered with epithelial cells with a self renewal potential to treat patients with complete ocular surface failure due to stem cell deficiency. Such a construct should not only replace the damaged tissue and minimise postoperative inflammation by restoring the integrity of the ocular surface, it should also take part in tissue regeneration by synthesis of new tissue by the transplanted cells and act as a scaffold to guide tissue

formation from the donor side (Kim and Mooney 1998). Various substrates ranging from natural biopolymers to artificial synthetic matrices have been suggested for corneal surface transplantation and could possibly be adapted for conjunctival reconstruction strategies. The substrate currently favoured for the optimisation of culture and transfer of epithelium onto the eye is amniotic membrane (AM, see chapter 1.3.4) (Tseng, Prabhasawat et al. 1998; Grueterich, Espana et al. 2002). However, supply of membranes can be challenging and its use requires donor screening that cannot completely eliminate the risk of transmission of viral agents. There have been only a limited number of attempts to design a synthetic substrate for the culture and delivery of limbal epithelial cells.

Pellegrini *et al.* expanded stem cells obtained from the limbal epithelium onto a fibrin substrate. This method has shown very promising results with the autografts successful in 14 of the 18 patients included in the study. At the end of the first month, the corneal surface was covered by a transparent, normal-looking epithelium and visual acuity improved from light perception or counting fingers to 0.8–1.0 (Rama, Bonini et al. 2001). To avoid cell damage from trypsin or Dispase II while detaching cells, Nishida *et al.* (Nishida, Yamato et al. 2004) have developed a temperature sensitive sol-gel-transition for the transfer of intact epithelial sheets to the cornea. For this technique a temperature sensitive surface was deposited onto a culture dish before oral mucosal epithelial cells were grown to a multilayer of cells on the dish. The cell sheet was then detached by changing the temperature. Such cell sheets were transplanted without suture to the ocular surface of 4 patients with total limbal

stem cell deficiency due to Stevens-Johnson syndrome or ocular cicatricial pemphigoid. Corneal transparency was maintained during a follow-up of 14 months and visual acuity improved although peripheral stromal vascularisation gradually recurred. The authors concluded that this type of ocular surface substitute might be an alternative to allogenic limbal transplantation for patients with severe bilateral disease (Nishida, Yamato et al. 2004). However, the oral mucosal cultures in these experiments still required the presence of bovine serum and animal feeder cells to attain confluence.

Studies of novel substrates have focussed on the growth and delivery of corneal epithelial cells in defined conditions excluding animal products, with surface chemistry manipulation of the carrier to allow adhesion and proliferation of the cells. The adhesion and proliferation of a human corneal epithelial cell line and mouse 3T3 fibroblasts on hydroxyl-terminated dendronized (perfectly branched polymers) surfaces has recently been reported (Benhabbour, Sheardown et al. 2008). The cells showed a greater affinity for the dendronized surfaces compared to the control gold surfaces at 24 hours. At longer incubation times, human corneal epithelial cell proliferation on the dendronized surfaces increased exponentially. Adhesion assays using serum-containing medium showed that cell attachment was diminished on PEG-grafted surfaces compared to the control surfaces (Benhabbour, Sheardown et al. 2008).

Acrylic acid polymer coated surfaces have been successfully used as a dermal epithelial cell culture substrate in serum-free conditions (Higham, Dawson et al. 2003; Sun, Higham et al. 2004; Bullock, Higham et al. 2006). Our group

adopted this approach and used an acrylic acid plasma polymerisation coating for the inner surface of a bandage contact lens to act as a carrier for limbal epithelial cells (Deshpande, Notara et al. 2009). The primary cultures of limbal epithelial cells were grown directly on the contact lens surface. This methodology provides a culture surface, a transport vehicle, a system for immobilising and protecting the cells on the eye. A range of plasma polymer coated surfaces were evaluated as substrates for serum-free culture of primary human limbal epithelial cells and the results suggest that these surfaces can be used successfully for the serum-free expansion of human limbal epithelial cells (Notara, Bullett et al. 2007). Substrate enhanced cell proliferation under serum-free conditions would mean a significant step towards a safer method for limbal epithelial SC delivery in the clinic.

1.3.2. Acellular polymers for matrix substitution

Severe conjunctival scarring and contraction is a major complication of chronic inflammatory ocular surface disease. The formation of scar occurs by the deposition, maturation and organization of newly synthesized collagen. Fibroblasts, the major source of collagen, become oriented in the wound bed and synthesize collagen fibers in a direction parallel to their orientation (Birk and Trelstad 1985). The uniaxial orientation of collagen in scar tissue is different from the random alignment of collagen fibers in normal connective tissues (Gabbiani, Hirschel et al. 1972).

To address the problem of conjunctival scarring, Hsu et al. examined the use of a porous collagen-glycosaminoglycan (CG) copolymer matrix as a graft for

8mm diameter full thickness conjunctival wounds in a rabbit model. Fornix depth was measured at day 1, 7, 14, 21 and 28 and compared to an ungrafted full thickness wound on the same eye. Compared to the ungrafted group, the presence of the CG copolymer significantly reduced fornix shortening at all time points. The copolymer degraded to approximately 45% of the initial area on day 14 and was almost completely degraded by day 28. The most active period of contraction was in the first 14 days after surgery and during that time the presence of α -smooth muscle actin (SMA) positive fibroblasts was noted (Hsu, Spilker et al. 2000), supporting the hypothesis that myofibroblasts actively participate in the wound contraction (Darby, Skalli et al. 1990). The authors referred to previous studies (Yannas 1998) and proposed that the random porous structure of the CG copolymer interrupts the alignment of myofibroblasts and collagen fibers and prevents their contraction (Hsu, Spilker et al. 2000). These results are similar to findings by Lee et al. who used a solvent-casting method to produce a porous poly(lactide-co-glycolide) (PLGA) scaffold that was then modified by collagen and hyaluronic acid (Lee, Oh et al. 2003). Eight mm diameter discs were then grafted into full-thickness conjunctival wounds in rabbits. After four weeks the grafted wounds showed significantly less fornix shortening compared to the controls and the grafts had not degraded. Similar to the previous study a random alignment of collagen fibers similar to normal conjunctiva was present, compared to ungrafted wounds which showed aligned collagen fibers typical of scar tissue.

Both of the previous studies demonstrate that transplantation of an engineered tissue substitute may reduce fornix shortening after injury by inhibiting scar

formation. This type of substitute may prove to be useful to cover small conjunctival defects, but as they both lacked an epithelium and took 14 days to epithelialize compared to 7 days for an ungrafted wound they cannot be used in clinical situations when donor epithelium is required. Additionally, they are not elastic enough to be transplanted into the fornix and it is unclear how fast they will degrade when there is ocular surface inflammation, e.g. with ocular cicatricial pemphigoid.

1.3.3. Collagen matrix with an epithelial layer

During embryogenesis, the proliferation and differentiation of epithelia are dependent on their associated mesenchyme (Dodson 1967). An epithelial cell *in vitro* will then only retain its proliferative potential and its differentiated phenotype under conditions that mimic its natural environment. The combination of a high cell density and an appropriate substrate in particular induce cell-cell and cell-matrix interactions (Watt 1986; Gurdon 1988; Matsumoto and Mooney 2006). Therefore the use of fibroblasts, extracellular matrix components or a basement membrane may help to maintain a progenitor cell population, which then can retain a differentiated epithelial phenotype and cell function *in vitro* and be desirable components of an engineered conjunctival substitute for the use in-vivo.

1.3.3.1. Extracellular matrix

The growth and differentiation of epithelial cells are limited when cells are cultured on conventional plastic or glass substrates (Oka, Perry et al. 1974;

Banerjee 1976; Topper and Freeman 1980). In a study by Tsai et al. the effect of type I collagen gel and matrigel on cell growth and differentiation were compared to conventional plastic and glass plates. Primary cultures on plastic reached confluence on day 7 and adopted a monolayer of small epithelial-like cells. However, upon subsequent subculture their growth rate reduced, the cells became enlarged and squamous, and exhibited obvious senescence. These morphological changes of senescence were even more striking in cultures grown on glass plates. In contrast, cells grown on collagen gel formed an organized monolayer, while on matrigel they showed a well organized globular growth pattern. Furthermore, the coated substrate systems promoted cell polarity, tight junction formation, and the development of a basement membrane. The authors concluded that growth and differentiation of epithelial cells was determined by their underlying extracellular matrix (Tsai and Tseng 1988).

1.3.3.2. Fibroblast co-culture and air-lifting

The growth of epithelial cell colonies and stratified epithelium is also enhanced by co-culture with fibroblasts (Rheinwald and Green 1975; Sun and Green 1977). In the conjunctiva epithelial cells grow on a meshwork of extracellular matrix substrate containing fibroblasts. To simulate this situation Tsai et al. cultured human conjunctival epithelial cells on a three dimensional collagen gel containing either normal human conjunctival fibroblasts or 3T3 mouse fibroblasts (3T3 feeder cells); an acellular collagen gel was used as a control. The epithelial cells cultured on the gel containing human fibroblasts grew

faster and formed larger colonies compared to cells grown on collagen gel without fibroblasts, while epithelial cell growth on the gel containing 3T3 cells was greatest. Sections from 2-week-old submerged specimens showed that epithelial cells on the human conjunctival fibroblast gel layer were mostly single to 2-cell layers, whereas the cells grown on the 3T3 containing gel had developed into 7 to 10-cell layers with evident stratification. When the epithelial cells were cultured at an air-liquid interface to simulate the normal ocular surface the number of cell layers in both groups was increased. Interestingly PAS positive cells, suggesting goblet cell differentiation, were only observed in epithelia cultured on the matrix containing human conjunctival fibroblasts. The authors concluded that a substrate containing a fibroblast/3T3 feeder layer was superior to an acellular substrate in supporting the proliferation and differentiation of conjunctival epithelial cells (Tsai, Ho et al. 1994).

1.3.4. Simple amniotic membrane transplantation

The amniotic membrane is the innermost layer of the placenta and consists of three layers: the epithelium, a thick basement membrane, and an avascular stroma. It may be used for protection only or as a substrate to support conjunctival cell migration and regeneration. It promotes rapid epithelialization, reduces inflammation and vascularization, and is capable of suppressing fibrosis (Shimazaki, Yang et al. 1997). Since the AM is thin and elastic it is well tolerated by the patient and the cosmetic result is superior to bulky mucous membrane grafts (Paridaens, Beekhuis et al. 2001). It has been used for fornix reconstruction following cicatrising conjunctivitis, irradiation, chemical or

thermal burns, resection of extensive conjunctival tumors or recurrent pterygia (Honavar, Bansal et al. 2000; Barabino and Rolando 2003; Barabino, Rolando et al. 2003; Solomon, Espana et al. 2003; Henderson and Collin 2008).

Reconstruction with AM can be successful but is prone to recurrent shrinkage if there is uncontrolled inflammation. Honavar et al. reported 10 patients with Stevens-Johnson syndrome in whom AM was used to reconstruct the corneal ocular surface and the conjunctival fornices. Over a mean follow-up of 14 ± 4 (range 9-30) months symblepharon recurred in 1 out of 10 eyes and entropion in 2 out of 7 lids. All patients reported an improvement in comfort and there was an improved visual acuity in 6 out of 9 eyes. Although 8 out of 10 eyes had recurrence of corneal vascularisation with a conjunctival epithelial phenotype suggestive of corneal limbal stem cell failure, this was less extensive than before surgery. Barabino et al evaluated AM for conjunctival reconstruction in 9 eyes (9 patients) with late stage ocular cicatricial pemphigoid. The symblephara had recurred after 28 weeks in 4 eyes. Although fornix depth increased from 2.8 ± 2.2 mm preoperatively to 5 ± 1.1 mm at 28 weeks about half of this additional fornix depth was lost within 4 months of surgery due to recurrent contraction. The procedure was not able to reduce conjunctival inflammation, but resulted in the recovery of a normal conjunctival epithelium with goblet cells in 6 eyes, thus supporting the hypothesis that AM is able to enhance conjunctival regeneration (Honavar, Bansal et al. 2000; Barabino and Rolando 2003; Barabino, Rolando et al. 2003). The success of fornix reconstruction with AM depends on the underlying disease. Solomon et al reported successful reconstruction in 12 out

of 17 patients and found that the outcome was best in eyes with symblephara following trauma. Fornix contraction due to an active inflammatory autoimmune disorder or recurrent pterygia showed a greater tendency to recur (Solomon, Espana et al. 2003).

Although mucous membrane (MM) is more bulky than AM there is less tendency for recurrence of symblepharon if it is used for fornix reconstruction. In a case control study Henderson et al. compared AM versus MM transplantation and found that 5 out of 6 eyes with AM but only 1 out of 6 with MM failed. It is assumed that the reason for the unsatisfactory outcome of AM grafts, especially in eyes with chronic inflammation, is continued inflammation and lysis of the AM by proteolytic enzymes on the ocular surface (Henderson and Collin 2008).

1.3.5. Amniotic membrane based cell sheets

1.3.5.1. AM carrier for expanded conjunctival epithelial cells

AM is a suitable carrier for the in-vitro culture of conjunctival cells and conjunctival epithelial cells grown on denuded AM develop a stratified epithelial phenotype (Meller and Tseng 1999). Epithelial differentiation and cell polarity are promoted by air-lifting (a method where the epithelial cells are grown on the air-liquid interface) and the cells do not express the cornea specific cytokeratins CK3 and CK12. Epithelium grown on AM under these conditions showed a non-goblet epithelial phenotype expressing ASGP1 and AMEM2 antigens (markers for non-goblet epithelial cells) but only occasional

cells positive for AM3 which recognizes conjunctival goblet cell-secreted mucins (Meller and Tseng 1999). Although goblet cell differentiation did not occur under these culture conditions in-vitro on AM the conjunctival progenitor cells for goblet and non-goblet cells were present. Meller et al. maintained cultures of conjunctival epithelium on AM for 14 days and implanted them subcutaneously into Balb/c athymic mice for a further 11 days. After this period the conjunctival cells on the AM showed PAS positivity and were immunoreactive for antibodies to conjunctival goblet cell mucin (MUC5AC, AM3) and glycocalyx (AMEM2). Slow cycling cells were identified by retention of BrdU labelling. The data suggests that conjunctival progenitor cells are preserved on AM, that they can differentiate into goblet cells in a permissive stromal environment (Meller, Pires et al. 2002), and that AM could be a suitable substrate for ex-vivo expansion of conjunctival progenitor cells prior to transplantation (Pellegrini, Traverso et al. 1997; Tsai, Li et al. 2000).

Culture systems both with and without human serum have been demonstrated to successfully maintain proliferation and differentiation of conjunctival epithelium cells (Risse Marsh, Massaro-Giordano et al. 2002; Ang, Tan et al. 2004; Ang, Tan et al. 2005). Ang et al cultured conjunctival epithelial cells on AM in serum-free medium and the cells became stratified, expressed the cyokeratins characteristic for non-keratinized stratified epithelium (CK4 and CK19), with goblet cell mucin detected by the presence of MUC5AC mRNA. Interestingly, the cells showed a greater proliferation capacity and stratification than cells cultured in serum containing medium, and airlifted cultures showed a reduced proliferation rate compared to submerged cultures. The authors

concluded that AM based conjunctival constructs grown in serum-free medium have the optimum proliferative and structural properties for clinical transplantation (Ang, Tan et al. 2004). In a further study by the same authors expanded autologous conjunctival epithelial sheets grown on AM were grafted in 22 patients after excision of primary pterygium. Almost immediate epithelialization occurred with earlier rehabilitation compared with transplantation of denuded AM. The effect was thought to result from reduced postoperative inflammation due to a viable surface epithelium (Ang, Tan et al. 2005).

1.3.5.2. Oral mucosal epithelial cell sheets

The potential of *in vitro* cultured autologous oral mucosal epithelium for ocular surface reconstruction is attractive because it is readily available and it is a similar stratified and non-keratinized epithelium. Epithelial cells isolated from the oral mucosa are thought to be at a lower stage of differentiation than skin keratinocytes. They have a faster cell turnover with resultant short culture time and they can be maintained in culture long-term without keratinisation (Hata, Kagami et al. 1995; Ueda, Hata et al. 1995).

When oral mucosal cells are cultured on AM the resultant construct has four to five layers of stratified and well differentiated epithelium without goblet cells. The cells have the characteristics of non-keratinized mucosa with expression of the cytokeratin 4/13 pair in the superficial and intermediate but not in the basal layers. Expression of keratin 3, present in the cornea, nose and some oral mucosa (Juhl, Reibel et al. 1989; Collin, Ouhayoun et al. 1992) was found

in all layers of the cultured oral mucosa cells, but no cornea specific keratin 12 was observed - indicating that the cells do not transdifferentiate into a corneal like phenotype (Nakamura, Endo et al. 2003). When these constructs were used as autografts in rabbits after superficial keratectomy the graft was clear and epithelialised after 10 days (Nakamura, Endo et al. 2003). Inatomi et al. evaluated the use of *in vitro* cultured oral mucosal cell sheets for ocular surface reconstruction in 15 eyes of 12 patients with Stevens-Johnson syndrome or chemical burns with an average follow-up of 20 months. The corneal surface was stable and transparent without any major complications in 10 of 15 eyes and visual acuity improved by more than 2 lines in 10 eyes (Inatomi, Nakamura et al. 2006).

Although AM-based oral mucosal cell sheets have so far only been applied for corneal surface reconstruction, they might also be a suitable for replacement of the conjunctiva.

1.4. Conclusions and aims

Regeneration of the conjunctiva is an essential part of ocular surface reconstruction, especially in patients with total surface involvement. In these patients attempts at corneal reconstruction alone will fail unless a deep fornix is restored (Barabino, Rolando et al. 2003; Espana, Di Pascuale et al. 2004; Tseng, Di Pascuale et al. 2005; Henderson and Collin 2008).

The functions of the conjunctiva are complex and therefore an ideal conjunctival substitute should meet the following criteria:

- 1. A flexible matrix with a good long-term stability and elasticity.**
- 2. A good biocompatibility and low immunogenicity.**
- 3. An epithelial layer with a self-renewal potential on the surface of the matrix, which contains both epithelial as well as goblet cells, essential for the tear film and ocular surface stability.**

The advantages of autologous tissues such as conjunctiva and oral or nasal mucosa are evident as they contain an appropriate matrix with epithelium and do not stimulate rejection. However this strategy is limited by the lack of suitable donor tissue when large grafts are required and these tissues may also be impaired in patients suffering from autoimmune cicatrising disease. Synthetic substitutes have been tested in animal models with promising initial results, but available synthetic substrates lack the mechanical properties to be used for fornix reconstruction. They also lack an epithelial layer and it is unclear how fast they will degrade if there is chronic inflammation.

Amniotic membrane meets many of the criteria of an ideal conjunctival tissue substitute. It is thin, elastic and it is not rejected by the host. Its usefulness for corneal and conjunctival reconstruction has been confirmed and it also a good substrate for the growth of epithelial cells. Amniotic membrane based cell sheets covered with limbal, conjunctival or oral mucosal epithelium have been successfully transplanted to the ocular surface. However, the results with

amniotic membrane grafts for conjunctival reconstruction in the presence of chronic inflammatory ocular surface disease are less satisfactory and it is important that new materials and methods are developed to address this problem.

Another area that needs improvement is the way epithelial cells are cultured and the problem of storage of cryopreserved epithelial cells to improve delivery of the cells to the patient. Current culture methods usually include the use of animal products like foetal calf serum, cholera toxin, bovine pituitary extract and 3T3 mouse feeder cells to expand epithelial cells *in vitro*. As the use of animal products always has the risk of disease transmission new complete animal free culture systems have to be developed. It also has to be examined if cryopreservation of these epithelial cells affects the progenitor cell population and thereby the quality of the graft delivered to the patient.

As only a small percentage of epithelial cells expanded *in vitro* show stem cell characteristics and the percentage of these cells decrease during *in vitro* expansion, new culture systems also need to improve the culture conditions for this subpopulation of progenitor cells to maintain and expand them *in vitro*.

Therefore aims for this thesis were:

1. To expand conjunctival epithelial cells *in vitro*, characterise the epithelial cell population and to evaluate the proportion of cells with progenitor cell characteristics.

2. To examine the effect of cryopreservation and long-term in vitro culture on the progenitor cell population of conjunctival epithelial cells.
3. To improve the maintenance of conjunctival epithelial progenitor cells during in vitro expansion by trying to mimic an environment in vitro which is more similar to the stem cell niche in vivo
4. To transfer the expanded conjunctival epithelial cells to different substrates as compressed collagen matrices and amniotic membrane and test the properties of these cell-matrix compounds with the ultimate goal to obtain a stable conjunctival substitute for conjunctival reconstruction, which then can be tested in a clinical trial.

Chapter 2:

Materials and Methods

2. Materials and Methods

2.1. Cell culture

2.1.1. Conjunctival research tissue

Conjunctival biopsies were taken from cadaveric human corneoscleral rims, surplus from surgery (obtained from Moorfields Eye Hospital Eye Bank). The tissue used had research consent given by the donor's next of kin, and this project had the appropriate ethics approval to use such tissue (Ethics Committee Approval Reference: 08/H0721/60).

2.1.2. Isolation and culture of conjunctival epithelial cells

Human conjunctival biopsies were obtained as follows. The corneoscleral rims were placed under a dissecting microscope and 2 to 3 conjunctival biopsies of approximately 2x2 mm were taken between 3-5 millimeters from the limbus (Fig. 6A). The biopsies were dissected from the underlying subconjunctival tissue, cut into 0.5-1 mm pieces and placed in T25 cell culture flasks on a 3T3 feeder layer. The cells were cultured in Conjunctival Epithelial Culture Medium (CECM) consisting of DMEM/F12 (1:1) (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Gibco), 0.1 nM cholera toxin (Sigma), 5 µg/ml human recombinant insulin (Sigma), 0.4 µg/ml hydrocortisone (Sigma), 0.18 mM adenine (Sigma), 2 nM triiodothyronine (Sigma), 5 µg/ml transferrin (Sigma) and 10 ng/ml epidermal growth factor (EGF) (Invitrogen). Culture medium was changed every other day and human

conjunctival epithelial cells (HCEC) were passaged upon reaching 70-80% confluence. When ready for passaging, feeders were removed from the culture using 1xtrypsin:EDTA, the epithelial cells remained attached to the tissue culture surface and were subsequently detached using 10x trypsin:EDTA. The harvested epithelial cells were quenched with CECM, centrifuged at 80-100g for 5 mins, and the cell pellet resuspended in CECM. HCEC's were cultured in the presence of 5% CO₂ in air at 37°C unless otherwise indicated.

2.1.3. Maintenance of 3T3 mouse fibroblasts for use as a feeder layer

The 3T3/J2 mouse fibroblasts were a gift from Fiona Watt and are referred to as 3T3 fibroblasts. These fibroblasts were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% Adult Bovine Serum (Gibco) and 1% penicillin/streptomycin (Gibco). Culture medium was changed three times a week and the cultures were passaged upon reaching 60-70% confluence at a ratio of 1:5. The cultures were maintained at 37°C and 5% CO₂ in air.

2.1.4. Growth arrest of 3T3 fibroblasts

3T3 fibroblasts were ready to be growth arrested when they reached 70-80% confluency. Fibroblasts were incubated with 4µg/ml Mitomycin C (MMC) (Sigma) in fibroblast culture medium for 2 hours at 37°C with 5% CO₂. They were then washed 3 times with Phosphate-Buffered Saline (PBS), and detached from the tissue culture flask using 1xtrypsin:EDTA. The cells were

then resuspended in fibroblast culture medium, and centrifuged at 80-100g for 5 minutes. The cell pellet was resuspended in fibroblast culture medium, cells counted and plated at a cell density of 2.4×10^4 cells/cm². Cells were allowed to attach for at least 2 hours, and the fibroblast culture medium replaced with CECM before seeding epithelial cells on top.

2.1.5. Visualisation of cell morphology using light microscopy

Cell morphology was monitored during cell culture using an inverted phase contrast microscope (Nikon Eclipse TS100 inverted phase contrast microscope, Nikon Instruments Europe B. V., Surrey, UK)

2.1.6. Cell counting with a haemocytometer

Cell numbers were counted using a haemocytometer (Neubauer-improved counting chamber, Marienfeld, Germany). Each chamber contains a grid of defined size. This grid consists of nine 1 x 1mm squares which are subdivided into a number of smaller sections. The chamber is 0.1mm deep and therefore each square has a defined volume. Each cell sample to be counted was well mixed, and a 10µl aliquot was then mixed with 10µl trypan blue (0.4%, Gibeco) and placed in a haemocytometer chamber. All trypan blue unstained living cells in the central 1 x 1mm square of the grid were counted, and the total number of living cells per ml calculated by multiplying the cell number by two and then by 10⁴. When counting cells, those which overlapped the top or right hand outer grid lines were counted but not those which overlapped the bottom

or left lines. The original sample was diluted if necessary to ensure the cell count was around 100. If diluted this was factored into the calculation. Cell counts were repeated to ensure that the sample aliquot counted was representative of the original cell suspension (Fig. 5).

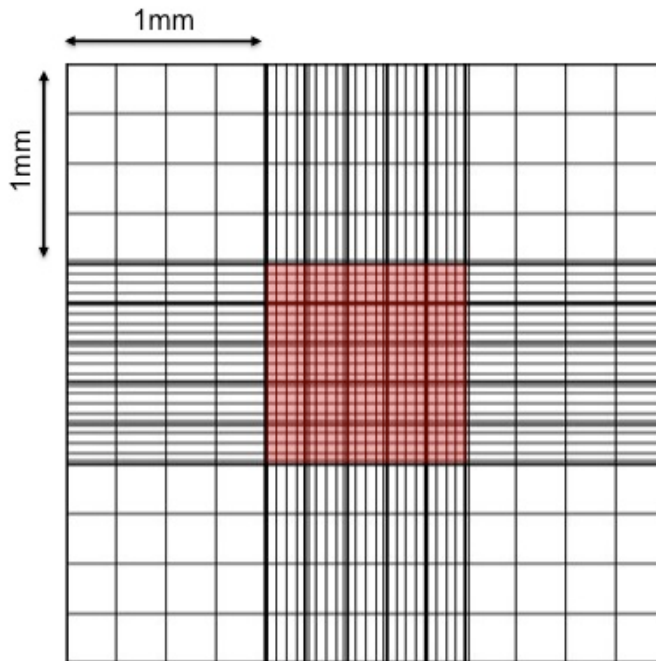


Figure 5: Haemocytometer grid for cell counting.

This grid contains nine 1 x 1mm squares which are divided into smaller sections. Counting the number of cells (of an undiluted sample) in the central square indicated in red and multiplying by 10^4 gives the number of cells/ml in the original sample.

2.1.7. Cryopreservation and thawing protocol for epithelial cells

The freezing medium consisted of 70% (v/v) CECM, 20% (v/v) FBS and 10% (v/v) dimethyl sulfoxide (DMSO). Directly after the transfer of the cells into the freezing medium, the samples were cooled in a controlled-rate freezing

container (Nalgene) designed to attain initial cooling at a rate of $-1^{\circ}\text{C}/\text{min}$ to -80°C and then transferred to liquid phase of liquid nitrogen and stored at -196°C . After cryostorage the cells were thawed rapidly within approximately 3-5 minutes under visual control at 37°C , immediately centrifuged to remove the DMSO and then resuspended in CECM.

2.1.8. Cell viability assessment

The viability of the epithelial cells before and after cryopreservation was assessed by vital staining with trypan blue (0.4%, Gibco). The number of trypan blue unstained living cells was counted before and after cryopreservation. The survival rate was calculated as the ratio between the number of living cells before and after cryopreservation.

2.2. General methods and assays

2.2.1. Colony forming efficiency assay

The colony forming efficiency (CFE) assay was used to give a measure of the ability of a cell population to found colonies. For the CFE assay, HCEC's were plated at a cell density of 500-1000 cells/well into six-well plates containing a growth arrested 3T3 feeder layer. This range of HCEC seeding densities was used to ensure that enough cells were seeded to generate colonies, but that not too many cells were seeded as colonies would merge in such cultures and make quantification difficult. Colonies need to be distinct from one another in this assay to enable the diameter and surface area of each colony to be

measured. The medium was changed on alternate days. The colonies were fixed between days 10-12 using cold methanol for 30 min at -20°C. Subsequently, the cells were re-hydrated with PBS and stained with a solution of 1% haematoxylin and 1% rhodamine. Finally, the plates were photographed on a lightbox. Image J software was used to count the total number of colonies and to measure the diameter and surface area of colonies with a smooth rounded perimeter. The total colony forming efficiency and the number of colonies with a surface area > 10mm² were calculated using the equation:

$$CFE(\%) = \frac{\text{number of colonies}}{\text{number of seeded cells}} \times 100$$

2.2.2. Clonal analysis

Colony-forming epithelial cells are heterogeneous in their capacity for sustained growth. Once a colony (clone) has been derived from a single cell, its growth potential can be estimated from the colony type formed by its cells after a single transfer to another plate. The clone can then be assigned to one of three types, namely holoclones, meroclones and paraclones (Barrandon and Green 1987). Holoclones are derived from a single stem cell and have the greatest reproductive capacity. Their cells can undergo 120-160 cell divisions (Pellegrini, Golisano et al. 1999). Colonies formed by a holoclone are large with smooth nearly circular perimeters and fewer than 5% of the colonies formed by the cells of a holoclone abort and terminally differentiate. Paraclones are formed by transient amplifying (TA) cells and contain exclusively cells with a short replicative lifespan (not more than 15 cell

generations), after which they uniformly abort and terminally differentiate. Paraclones have an irregular perimeter. The third type of clone, the meroclone, contains a mixture of cells of different growth potential and is a transitional stage between the holoclone and the paraclone (Barrandon and Green 1987). For the clonal analysis 200 single epithelial cells/well were seeded into six well plates containing a 3T3 feeder layer. After seven days of culture colonies generated from single cells were located using an inverted phase contrast microscope. The colonies were trypsinised using cloning rings and transferred to a second dish. The secondary colonies, which again had developed from single cells, were fixed and stained between days 10-12 using the same method as in 2.2.1.

2.2.3. Immunocytochemistry

2.2.3.1. Cell fixation

Prior to fixation cells were washed 3 times with PBS, and incubated in 2% paraformaldehyde for 15 minutes at room temperature. Paraformaldehyde was removed and cells washed with PBS before the immunostaining procedure. If plates were to be immunostained at a later date, cells were incubated with 20% sucrose at room temperature for 15 minutes. Sucrose was then discarded and cells thoroughly air-dried in the tissue culture hood for several hours. Chamber slides were sealed with parafilm and stored at -20°C until ready for staining.

2.2.3.2. Immunostaining protocol

Four-well and eight-well permanox chambered slides (Labtek, Nunk) were used for immunocytochemical analysis. Cells were washed 3 times with PBS and the cultures were blocked for 1h in PBS supplemented with 5% goat serum or donkey serum according to the species of the respective primary antibody (Sigma) and 0.5% Triton X100 (Sigma), followed by the primary antibody or normal mouse/rabbit/goat IgG (Santa Cruz Biotechnology) (negative control) overnight at 4°C.

Subsequently, the cells were incubated with their respective secondary antibody (donkey anti-goat TRITC, goat anti-mouse FITC, goat anti rabbit TRITC or FITC antibodies respectively (Alexa Fluor)), washed and counterstained with FITC or TRITC conjugated phalloidin (Sigma). Finally, the chamber slide wells were removed and the slides were mounted using Vectashield medium with or without DAPI (Vector Labs). All incubations apart from the primary antibody incubation were performed at room temperature, and each step was interspersed with three 5 minute rinses with PBS containing 0.5% Tween-20 (Sigma).

2.2.4. Immunohistochemistry

Human conjunctival biopsies were fixed in 4% paraformaldehyde (PFA) for 1 hour. To obtain cryostat sections, the tissue was cryoprotected with 30% sucrose dissolved in PBS overnight prior to embedding in O.C.T. (optimum cutting temperature) compound (VWR, U.K.) and rapidly frozen over dry ice-

cold acetone. Conjunctival sections were obtained using a Leica CM1850 cryostat (Leica Microsystems (UK) Ltd). The sections were cut to a thickness of 15-20µm, placed on superfrost plus slides and allowed to dry at RT for approximately 1 hour. The slides were then stored at -80°C until required for immunostaining. The Immunostaining was performed as described under section 2.2.3.2.

| Primary Antibody (raised in) | Details | Dilution used |
|------------------------------|-------------------------------------|---------------|
| CK19 (mouse) | M0888 (DAKO) | 1:100 |
| CK15 (mouse) | SC-47697 (Santa Cruz Biotechnology) | 1:100 |
| CK12 (goat) | SC-17101 (Santa Cruz Biotechnology) | 1:100 |
| CK4 (mouse) | 10525 (Cappel/Millipore) | 1:50 |
| p63 α (rabbit) | 4892 (Cell Signaling) | 1:150 |
| ABCG2 (mouse) | MAB4146 (Millipore) | 1:200 |
| Ki67 (rabbit) | AB9260 (Chemicon/Millipore) | 1:250 |
| MUC5AC (mouse) | AB24070 (Abcam) | 1:200-1:400 |
| β -Catenin (rabbit) | AB6302 (Abcam) | 1:1000 |
| Cyclin D1 (rabbit) | AB16663 (Abcam) | 1:100 |
| GAPDH (mouse) | MAB374 (Millipore) | 1:500 |

Table 1: Primary antibodies for immunostaining and westernblot.

2.2.5. Histological staining of paraffin sections

Conjunctival biopsies were fixed in 4% PFA for 1 h at room temperature before embedding in paraffin for sectioning. A microtome was used to cut 5-8 mm

sections, which were placed on superfrost slides. After rehydration through a series of alcohols to water, representative sections were stained with haematoxylin and eosin (H & E) or periodic acid-Schiff (PAS) and then mounted and coverslipped using DPX mounting medium. Sections were imaged using an Olympus BX50 light microscope and Evolution MP color camera (Media Cybernetics).

2.2.6. Semi-quantitative RT-PCR

Total cellular RNA was isolated from epithelial cell pellets using the RNeasy system (Qiagen, Germany) according to the manufacturer's instructions. Residual genomic DNA was eliminated from the samples by On-Column DNase digestion (RNase-Free DNase Set, Qiagen, Germany). For the RT-reaction, 1 µg of total RNA was reverse-transcribed in 20-µl reactions consisting of 5 mM MgCl₂, 1 mM deoxynucleoside-5'-triphosphate (dNTP), 1 U/µl RNase inhibitor, 0.8 U/µl AMV reverse transcriptase (Roche Diagnostics, Switzerland), and 80 ng/µl oligo(dT)-15 primers (Roche) in 10 mM Tris/HCl buffer (pH=7.4) containing 50 mM KCl. The mixture was incubated as follows: 10 minutes at 25°C, 60 minutes at 42°C, 5 minutes at 99°C, and 5 minutes at 4°C in a thermal cycler (Eppendorf AG, Germany). Polymerase chain reaction (PCR) amplification was then performed; the amplification was performed in a final volume of 14 µl by addition of 10µl of MegaMix Blue (Helena Biosciences). The mixture was initially incubated at 94°C for 2 minutes followed by 30–37 cycles under the following conditions: 94°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 1 minute, and one cycle of

72°C for 5 minutes. A “no-RT” control was performed to confirm the absence of genomic DNA. PCR products were analyzed by agarose gel electrophoresis (2%) containing gel-Red (Cambridge Biosciences). Primer sequences for all studied genes are given in Table 2.

| Gene | Sense Primer | Antisense primer |
|--------------------------------|------------------------------------|--|
| ABCG2 (human) | TGCAACATGTACTGGCGAAGA | TCTTCCACAAGCCCCAGG |
| Δ Np63 α (human) | GGAAAACAATGCCCAGACTC (Δ N) | ATGATGAACAGCCCCAACCTC (α -termini) |
| CK15 (human) | GGAGGTGGAAGCCGAAGTAT | GAGAGGAGACCACCATCGCC |
| MUC5AC (human) | TCCACCATATACCGCCACAGA | TGGACCGACAGTCACTGTCAAC |
| GAPDH (human) | GCCAAGGTCATCCATGACAAC | GTCCACCACCCTGTTGCTGTA |

Table 2: Primer sequences for semi-quantitative RT-PCR

2.2.7. Western blot analysis

Protein was extracted from cells using RIPA buffer (Pierce) and quantified using a BCA protein assay kit (Pierce Biotechnology) in accordance with the manufacturer's instructions. Reducing agent (Invitrogen) and loading buffer (Invitrogen) were added to 40 μ g of each protein sample. Samples were then boiled at 70°C for 10 minutes before running on a 10% bis-tris gel. Magic Mark XP Western Protein Standard (Invitrogen) was also loaded onto the gel to allow molecular weight estimation. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for

30 minutes at room temperature in blocking solution: tris-buffered saline with 0.1% Tween20 (TBST) containing 5% dried milk (nonfat) and 2% fetal bovine serum. The membrane was then incubated with primary antibody diluted in blocking buffer at 4°C overnight, or for 2 hours at room temperature on a shaker. The membrane was then washed for 3x 20 minutes with TBST, before incubating with the secondary antibody diluted in blocking buffer for 1 hour at room temperature. The membrane was again washed for 3x 20 minutes with TBST before visualisation with enhanced chemiluminescence reagent (Amersham Biosciences) and a Fuji image reader (LAS-1000 Pro version 2.1). Secondary antibodies used were goat anti-mouse HRP (Dako) and goat anti-rabbit HRP (Dako). Secondary antibodies were used at a dilution of 1:5,000 to 1:10,000.

2.2.8. Calculation of cell population doublings

The cell generation number, n , (population doubling) was calculated using the following equation: $n = (\log Y - \log X) / 0.301$, where Y is the final cell count and X is the number of clonogenic cells (Rheinwald and Green 1975; Pellegrini, Golisano et al. 1999; Freshney 2000). Clonogenic cells were calculated from the colony forming efficiency data, which was determined separately for every passage. All colonies were evaluated whether progressively growing or aborted as described above.

2.2.9. Statistical Analysis

Statistical analysis of results was carried out using Prism 5.0 software (GraphPad, USA). For two group comparison, the Student's unpaired t-test was used and for multiple group comparison, the one-way Analysis Of Variance (ANOVA) with Bonferroni post-hoc tests was employed. Sets of data producing $p < 0.05$ were considered statistically significant. All experiments were repeated three times, if not stated otherwise. All error bars represent standard deviation of the mean unless otherwise stated.

Chapter 3:

**Characterisation and expansion of the
conjunctival cell population *in vitro***

3. Characterisation and expansion of the conjunctival epithelial cell population *in vitro*

3.1. Introduction

Different methods have been described for the cultivation of conjunctival epithelial cells *in vitro*. Pellegrini et al reported the use of conjunctival epithelial single cell suspensions, which were cultured in the presence of 3T3 fibroblasts in foetal calf serum containing medium (Pellegrini, Golisano et al. 1999), a method originally described by Green et al. for epidermal epithelial cells (Rheinwald and Green 1975) (cell suspension method). A different method describes the use of conjunctival tissue explants obtained from conjunctival biopsies (explant method). The conjunctival explants were cultured with and without 3T3 fibroblasts and with and without foetal calf serum (Diebold, Calonge et al. 1997; Risse Marsh, Massaro-Giordano et al. 2002; Ang, Tan et al. 2004). Also methods to culture conjunctival single cell suspensions or conjunctival explants directly on amniotic membrane or collagen gels have been reported (Tsai, Ho et al. 1994; Meller, Dabul et al. 2002; Ang, Tan et al. 2004).

The use of the “explant method” offers the advantage that only a very small biopsy is needed for the expansion of the conjunctival epithelial cells. In a clinical setting, where biopsies are obtained from patients for *in vitro* epithelial cell expansion and transplantation the amount of tissue that can be biopsied is very limited and therefore it was decided to use this method for epithelial cell expansion.

Aim of the first experimental series was to expand human conjunctival epithelial cells from biopsies using the explant method and to characterise the conjunctival epithelial cell population *in vitro*.

3.2. Methods

The methods used for the experiments in this chapter are described in chapter 2 (Materials and Methods). Conjunctival cells from six conjunctival biopsies from six different donors were used for the experiments. The CFE-experiments and the clonal analysis were conducted with cells from four different donors and with three replicates per experiment.

3.3. Results

3.3.1. Characterisation of the human conjunctival biopsies

Characterisation of conjunctival tissue sections from the biopsy location by immunocytochemistry, showed strong immunoreactivity for CK19 in all cell layers (Fig. 6B). CK4 positivity was also found in all epithelial layers, but staining of the basal layer was much weaker and more focal (Fig. 6C, arrowhead). No immunoreactivity for the cornea specific marker CK12 was observed (Fig. 6D).

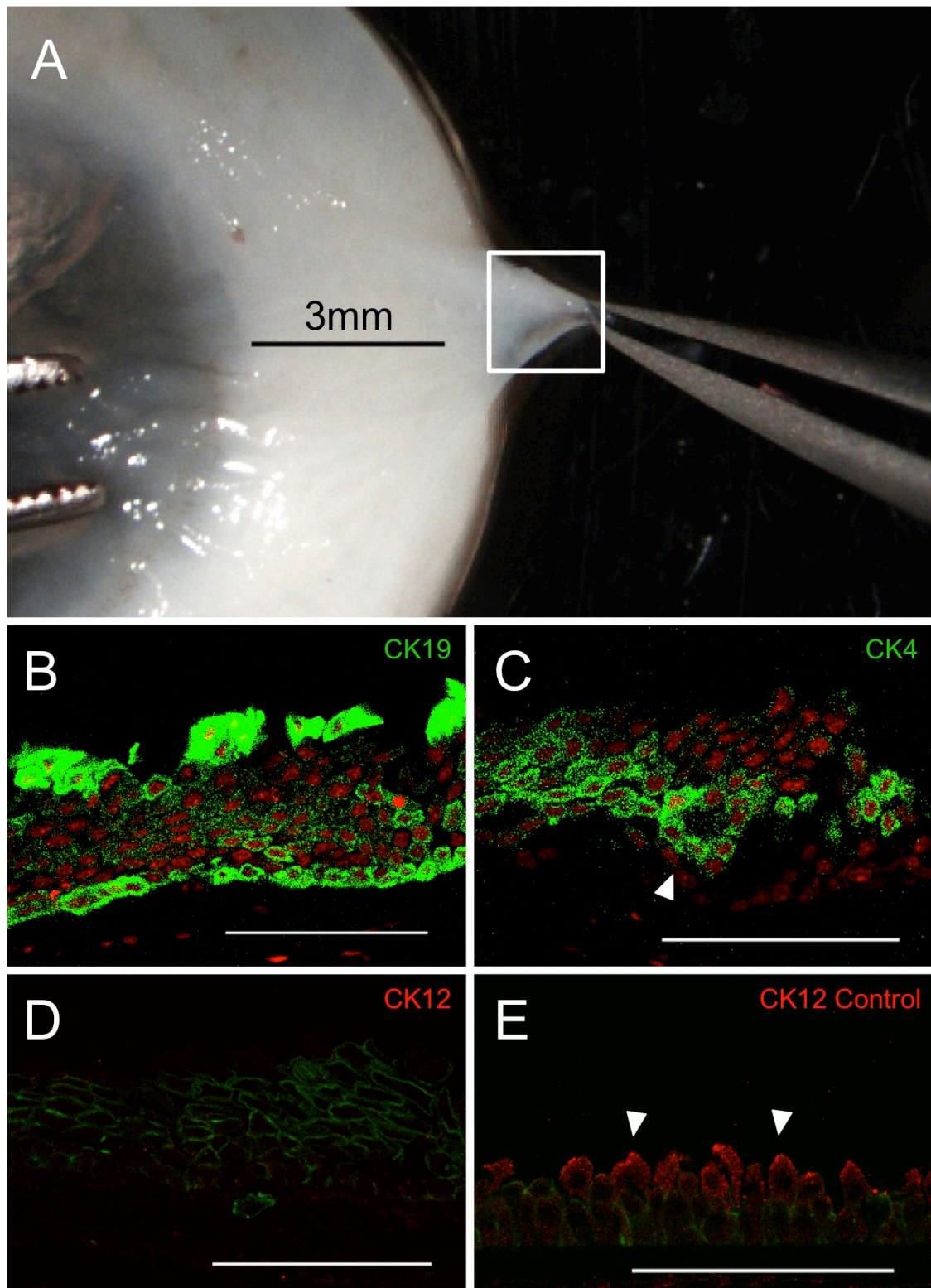


Figure 6: Conjunctival biopsy location and characterization.

Conjunctival biopsy location (square), 3mm away from the limbus (A). Conjunctival tissue sections from the biopsy side showed strong

immunoreactivity for CK19 (green) in all cell layers (B). CK4 (green) positivity was also found in all epithelial layers, but staining of the basal layer was much weaker and more focal (C, arrowhead). No immunoreactivity for the cornea specific marker CK12 (red) was observed (D). E: Control section from the central cornea showing strong CK12 (red) positivity (arrowheads). The sections were counterstained with propidium iodide (red, B+C) or phalloidin-FITC (green, D+E). Scale bars, 100 μ m.

3.3.2. Cell culture model for conjunctival epithelial cells

After the minced biopsy was placed on a culture dish (Fig. 7A), the outgrowth from the conjunctival explants started between day 2 and 5 (Fig. 7B) and the cells became 70-80% confluent after approximately 2 weeks. The cells from P₀ culture exhibited a cuboidal shape with a high nuclear to cytoplasmic ratio (n/c) (Fig. 7C).

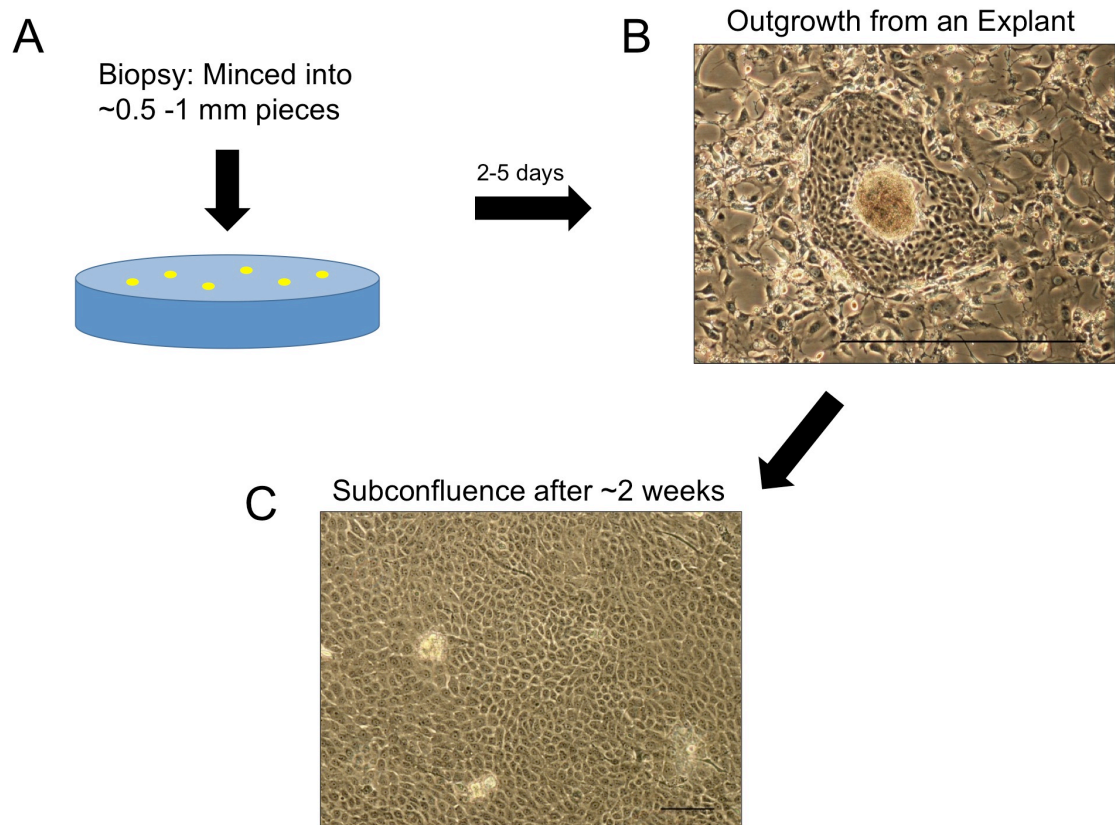


Figure 7: Cell culture model for conjunctival epithelial cells.

The biopsies were placed on a culture dish in presence of a growth arrested 3T3 feeder layer (A). Outgrowth from the conjunctival explants started between day 2-5 (B) and subconfluence of the P_0 culture was reached after approximately 2 weeks. Scale bars: 1 mm (B), 100 μ m (C).

3.3.3. Characterisation of the conjunctival epithelial cell population *in vitro*

Characterisation of P_0 cultures of the conjunctival epithelial cells revealed strong immunoreactivity for the conjunctival marker CK19 (Fig. 8A) and no immunoreactivity for the cornea specific marker CK12 (Fig. 8B).

The epithelial cells showed strong reactivity for the putative progenitor cell marker p63 α (Fig. 8D). Also the ATP-binding cassette transporter protein

ABCG2, was strongly expressed, where as expression was stronger in cells with a high n/c ratio (Fig. 8E, Arrowheads). Co-localisation of CK15, another putative progenitor cell marker and p63 α revealed a subpopulation of CK15 positive cells within the population of p63 α positive cells (Fig. 8F, Arrowheads).

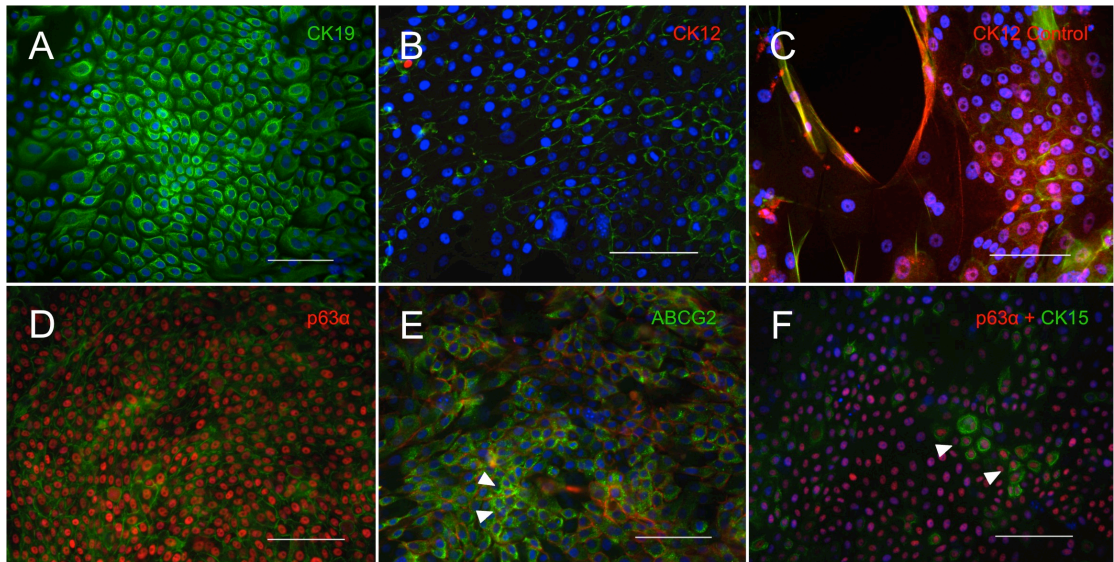


Figure 8: Conjunctival epithelial cells characterization.

Conjunctival epithelial cells showed strong immunoreactivity for CK19 (green, A), but no immunoreactivity for the cornea specific marker CK12 (red, B). C: Control slide with cultured cells from the central cornea showing strong CK12 (red) positivity. The epithelial cells also expressed p63 α (red, D) and ABCG2 (green, E). Co-localisation of CK15 (green) and p63 α (red) revealed a subpopulation of CK15 positive cells within the population of p63 α positive cells (F, arrowheads). The cultures were counterstained with a vectashield medium with DAPI (blue) and some with phalloidin-FITC (green, B, C, D) or TRITC (red, E). Scale bars, 100 μ m.

3.3.4. Colony forming capacity and clonal analysis

Conjunctival epithelial cells obtained from the explant culture (P_0) showed a high total colony forming efficiency of $8.78 \% \pm 3.40 \%$ (Mean \pm SD). These colonies exhibited smooth perimeters and contained small tightly packed cells. Evaluation of the surface area of the colonies revealed that $1.11 \% \pm 0.32 \%$ of the colonies had a surface area $> 10\text{mm}^2$, a characteristic of holoclone colonies (Barrandon and Green 1987) (Fig. 9A). When colonies generated from single cells were trypsinised and transferred to another dish after seven days of culture, they were capable of producing holoclone-, meroclone- and paraclone- colonies (Fig. 9B).

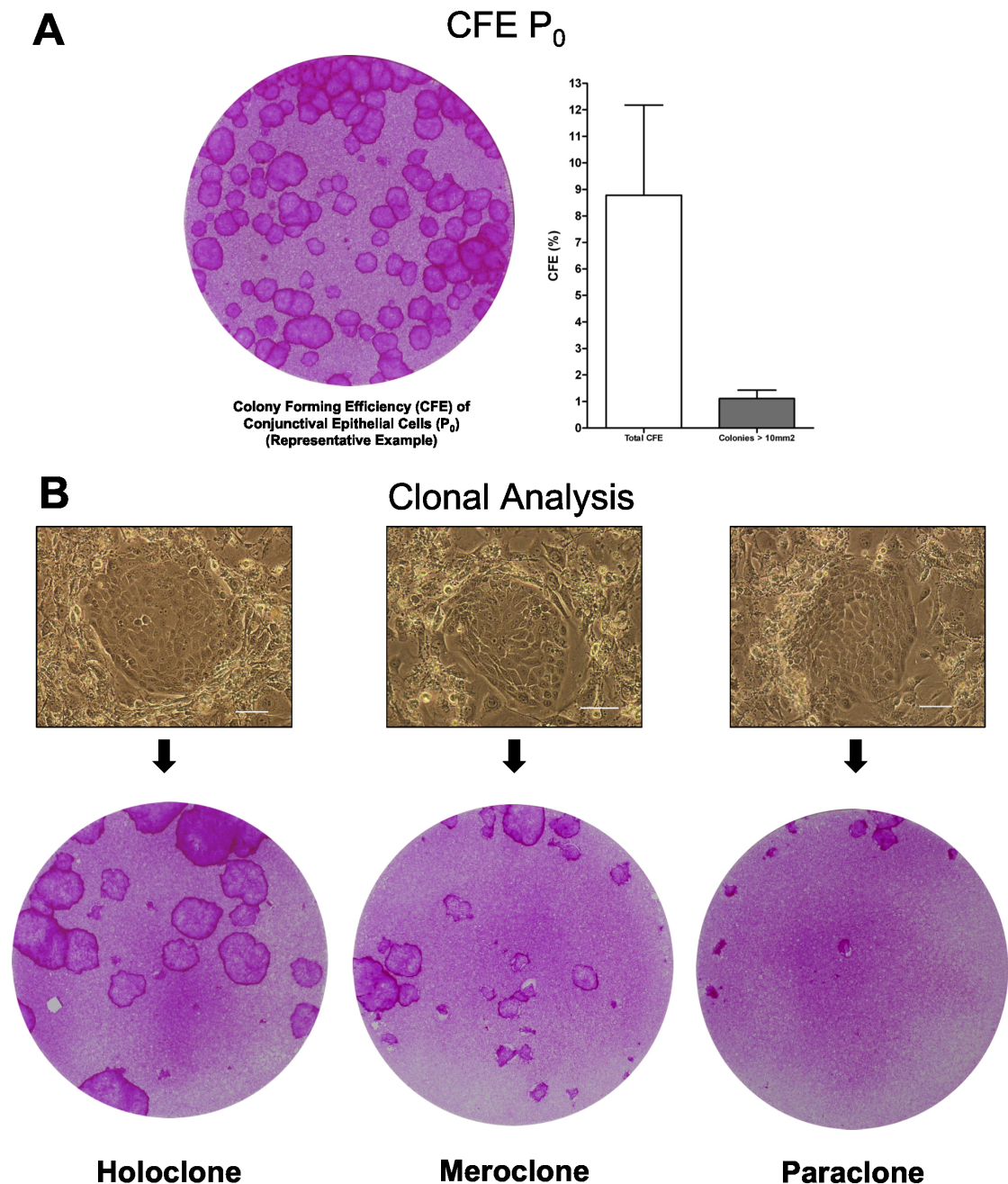


Figure 9: Colony forming efficiency and clonal analysis from conjunctival epithelial cells grown out of explants culture.

A: Conjunctival epithelial cells from the explant culture (P₀) showed a high total colony forming efficiency of 8.78 % ± 3.40 % (Mean ± SD). The colonies exhibited smooth perimeters and contained small tightly packed cells. Evaluation of the surface area of the colonies revealed that 1.11 % ± 0.32 % of the colonies had a surface area > 10mm². **B:** When colonies

generated from single cells were trypsinised and transferred to a second dish after seven days of culture, they were capable of producing holoclone, meroclone and paraclone colonies.

3.4. Discussion

The results confirmed that human conjunctival epithelial cells can be successfully grown out of biopsies using an explant culture method. The characterisation by immunocytochemistry showed that the cultured epithelial cells grown out of the explants, strongly expressed CK19, a marker for conjunctival cells, which is also expressed by conjunctival cells *in vivo*. The epithelial cells showed no expression of the corneal cell marker CK12.

When epithelial cells are expanded *in vitro* from small biopsies it is also important to know whether cells with progenitor cell characteristics migrate from their “niche” environment out of the biopsy onto the culture dish. There is evidence that epithelial progenitor cells can migrate out of an explanted biopsy (Li, Hayashida et al. 2007) but it has also been shown that stem cell properties from epithelial cells decrease with the distance from the original explant and that the proximity of the stem cell population to their original niche environment in the explant might be important in maintaining the stem cell population in an undifferentiated stage (Kolli, Lako et al. 2008).

In this study, conjunctival epithelial cells grown out of explants exhibited a cuboidal shape with a high nuclear to cytoplasmic ratio, indicating that many of the cells remained in a relatively undifferentiated stage, a characteristic of epithelial progenitor cells. Also strong expression of the putative progenitor cell

markers p63 α , ABCG2 and CK15 was found. Additionally the epithelial cells demonstrated a high total colony forming efficiency of 8.78 % \pm 3.40 % and evaluation of the surface area of the colonies revealed that 1.11 % \pm 0.32 % of the colonies had a surface area > 10mm², a characteristic of holoclone colonies (Barrandon and Green 1987). When colonies generated from single cells were trypsinised and transferred to a second dish after seven days of culture, they were capable to produce holoclone, meroclone and paraclone colonies. Holoclones are thought to be generated by stem cells (Barrandon and Green 1987; Pellegrini, Golisano et al. 1999) and the generation of holoclone colonies from cells grown out of explant cultures supports the concept that progenitor cells can migrate from the explants onto the culture dish.

In conclusion, this experimental series shows that conjunctival epithelial cells can be successfully expanded from biopsies using an explant culture method. Additionally, it indicates that conjunctival epithelial cells, grown out of explants can produce Holoclone, Meroclone and Paraclone colonies, suggesting that this epithelial cell population contains a subpopulation of cells with characteristics of epithelial progenitors.

Chapter 4:

**Evaluation of the effect of long-term *in vitro*
culture and cryopreservation on
conjunctival epithelial progenitor cells**

4. Evaluation of the effect of long-term *in vitro* culture and cryopreservation on conjunctival epithelial progenitor cells

4.1. Introduction

Transplantation of cultivated conjunctival epithelial cells on amniotic membrane for conjunctival replacement has already been used in the clinic for patients with non-chronic inflammatory disorders such as leaking blebs after glaucoma surgery and conjunctival replacement after pterygium surgery (Tan, Ang et al. 2004; Ang, Tan et al. 2005). However a shortage of human donor tissue and the tendency of epithelial cells to differentiate if they are held too long in culture before transplantation are major problems that limit the availability of tissue engineered epithelial cell sheets. The ability to expand and store conjunctival cells prior to clinical use and maintain their stem cell population would be an advance. A variety of cryopreservation methods have been developed to maintain the viability of various cells such as blood cells (De Bruyn, Delforge et al. 2003), spermatozoa (Polge, Smith et al. 1949) and epithelial cells (Harkin, Barnard et al. 2004; Kito, Kagami et al. 2005; Oh, Kim et al. 2007; Yeh, Yao et al. 2008). However the effects of cryopreservation and long-term *in vitro* culture on the progenitor cell characteristics of epithelial cells have not been assessed yet. The aim of this study was to evaluate whether and how cryopreservation and long-term *in vitro* expansion alter progenitor cell characteristics and proliferative capacity of conjunctival epithelial cells.

4.2. Methods

The methods used for the experiments in this chapter are described in chapter 2 (Materials and Methods). Conjunctival cells from seven conjunctival biopsies from seven different donors were used for the experiments. Cells from four different donors were serially cultivated and three replicates were performed per CFE-experiment. The methods specific to this chapter are described below.

4.2.1. Experimental setup for the cryopreservation experiments

For the cryopreservation experiments, the conjunctival epithelial cells from the primary culture (P_0) were passaged when they reached approximately 70-80% confluence. An aliquot with half of the cells from the initial culture was cryopreserved and stored in the liquid phase of liquid nitrogen at -196°C for 14 days as described in section 2.1.7. The cells were initially seeded at a density of $2.2\text{-}5.0 \times 10^5$ cells depending on the yield of the P_0 culture and were always passaged in a ratio of 1:2. Non-cryopreserved cells and cells cryopreserved for 14 days were serially cultured until passage 4. From every passage, cells were analysed by immunocytochemistry, a colony forming efficiency assay was performed and RNA was isolated for semiquantitative RT-PCR. Additionally conjunctival donor epithelial cells were cryopreserved for more than 6 months (202.7 ± 13.0 days) and then cell viability, putative stem cell marker expression and colony forming efficiency were compared with the other two groups at P_0 (Fig. 10).

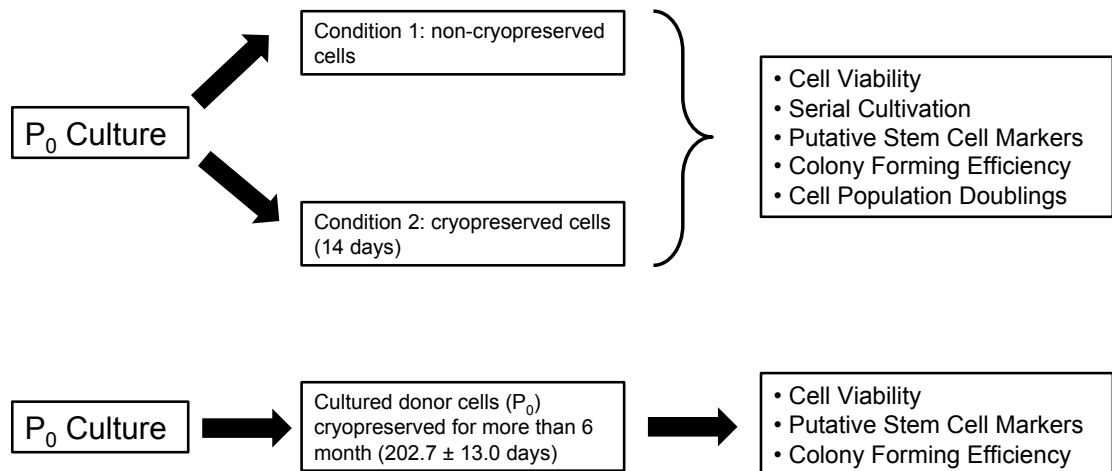


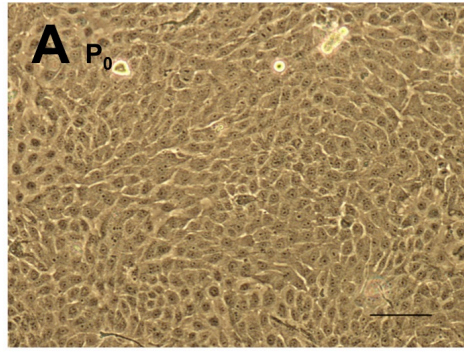
Figure 10: Experimental sequence for the cryopreservation experiments. An aliquot with half of the conjunctival epithelial cells from the initial culture (P_0) was cryopreserved for 14 days and cell viability, putative stem cell marker expression, colony forming efficiency and cumulative cell population doublings were then compared to the non-cryopreserved epithelial cells from the same donor. Additionally conjunctival donor epithelial cells were cryopreserved for more than 6 months (202.7 ± 13.0 days) and then cell viability, putative stem cell marker expression and colony forming efficiency were compared to the other two groups at P_0 .

4.3. Results

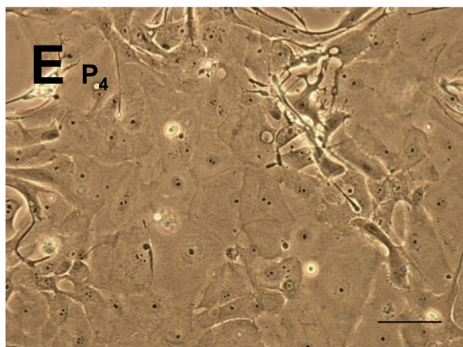
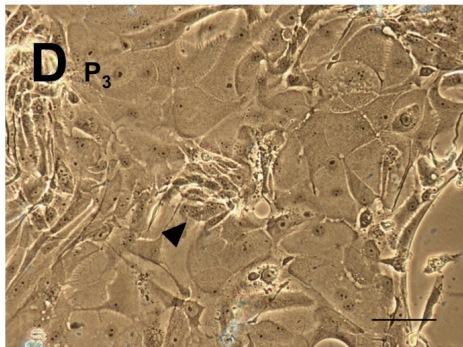
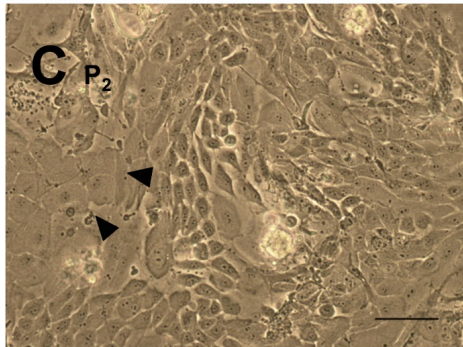
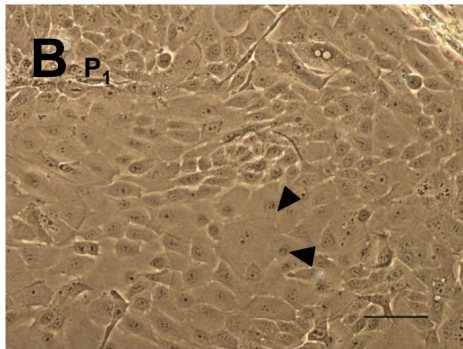
4.3.1. Morphology of conjunctival epithelial cells during long-term *in vitro* expansion

The conjunctival epithelial cells from the P_0 culture exhibited a cuboidal shape with a high nuclear to cytoplasmic ratio (n/c) (Fig. 11A). At P_1 the majority of the cells were still small and cuboidal, but flattened cells with a lower n/c ratio

were also noted (Fig. 11B+F, Arrowheads). Between P₂ and P₃ more enlarged cells were present in the cultures indicating differentiation (Figure 11C+G, Arrowheads), but areas of smaller cells were still noted at P₃ (Figure 11D+H, Arrowheads). At P₄ the majority of cells exhibited a flattened shape with a low n/c ratio. In most cases cultures reached senescence at P₄ (Figure 11E+I). No difference was noted between the morphology of non-cryopreserved (Fig. 11B-E) and cryopreserved (Fig. 11F-I) conjunctival epithelial cells at all passages.



Non-cryopreserved



Cryopreserved 14d

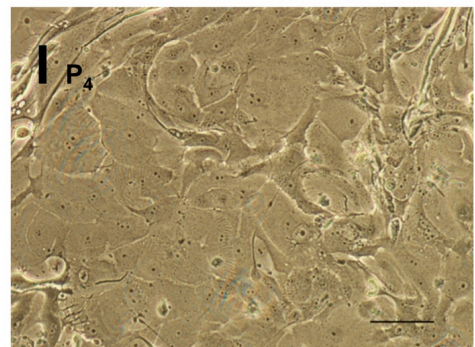
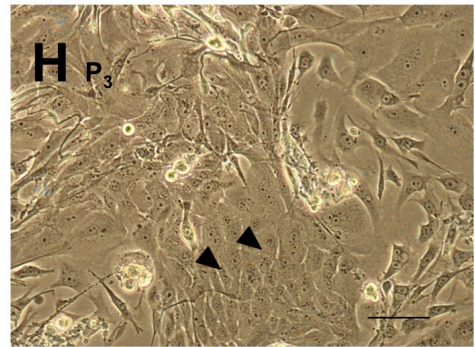
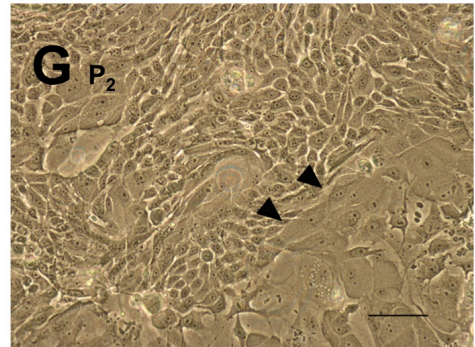
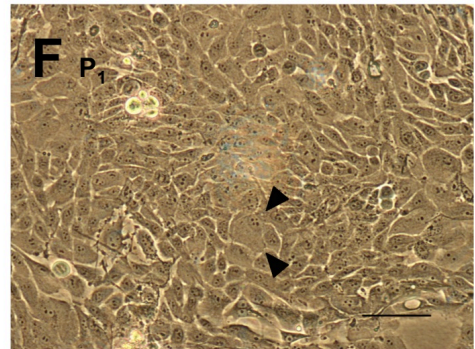


Figure 11: Morphology of non-cryopreserved (B-E) and for 14 days cryopreserved conjunctival epithelial cells (F-I) during serial cultivation.

A: Conjunctival epithelial cells from passage 0 (P₀). The epithelial cells exhibit a cuboidal shape with a high n/c ratio. B+F: P₁-culture showing a majority of small and cuboidal, but also some flattened cells (Arrowheads). C+G: P₂-culture, still exhibiting many small cuboidal cells, but also larger numbers of more differentiated looking cells (arrowheads). D+H: P₃-culture, showing large numbers of flattened cells and small islands of undifferentiated looking cells (Arrowheads). E+I: P₄-culture with a majority of cells exhibiting a flattened shape with a low n/c ratio. Scale bars: 100 µm.

4.3.2. Immunohistochemistry and semi-quantitative RT-PCR

Cultured conjunctival epithelial cells were characterised by immunostaining at P₁. Both, non-cryopreserved and cryopreserved cells showed strong positivity for CK19 (Fig. 12A-C). Immunostaining for CK4 exhibited strong positivity in differentiated, flattened cells, whereas the majority of small cuboidal cells were CK4 negative (Fig. 12D-F). Immunostaining for p63α showed strong positivity especially in small cuboidal cells with a high n/c ratio (Fig. 12G-I) and the conjunctival epithelial cells also exhibited immunoreactivity to ABCG2 (Fig. 12J-L). No difference in expression of CK19, CK4, p63α and ABCG2 was noted between non-cryopreserved cells and cells cryopreserved for 14 and 202.7 ± 13.0 days.

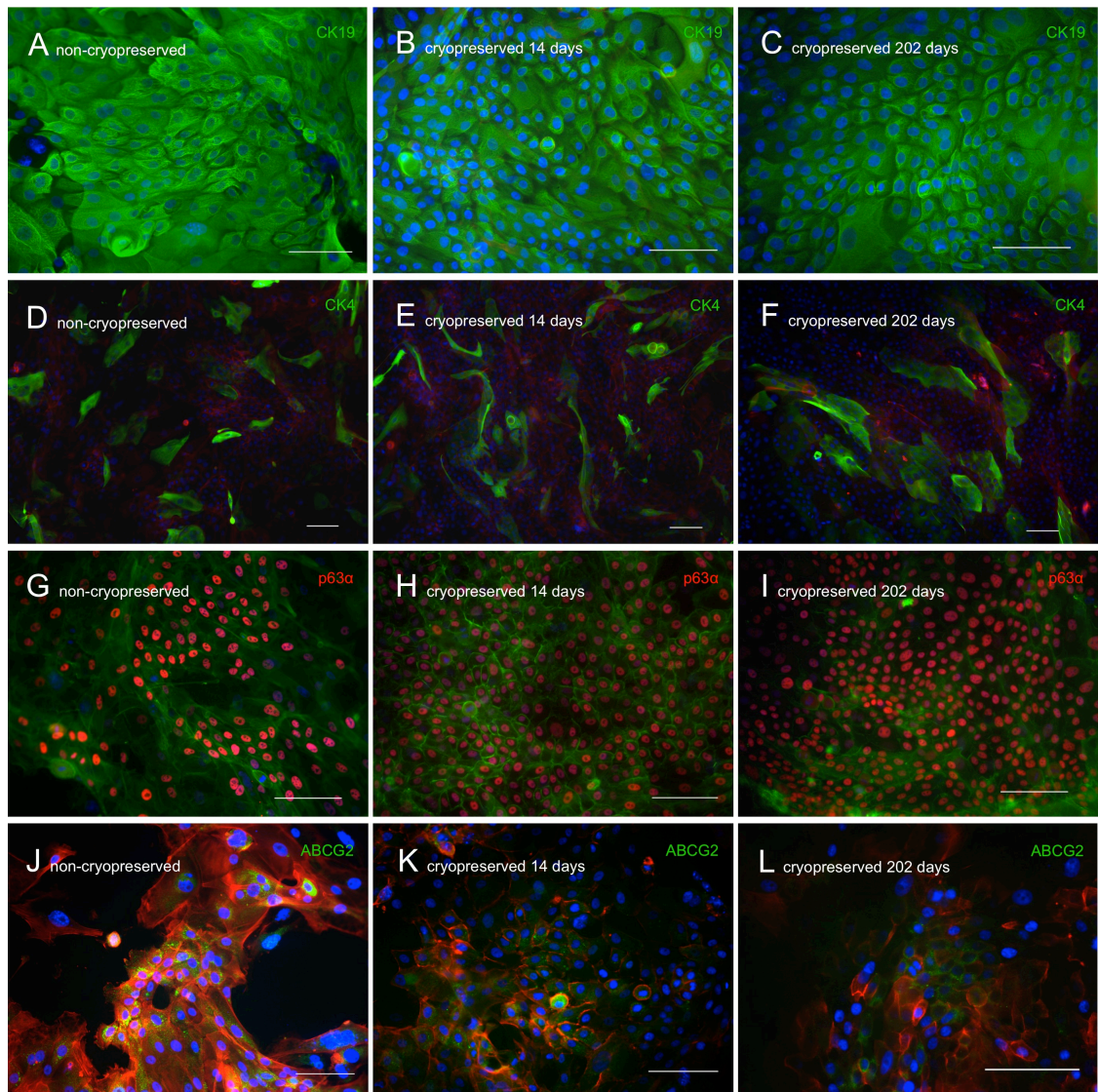


Figure 12: Expression of cytokeratins and putative progenitor cell-like markers at P₁ as assessed by immunostaining.

Conjunctival epithelial cells expressed CK19 (green, A-C), CK4 (green, D-F), p63 α (red, G-I) and ABCG2 (green, J-L). No difference in marker expression was observed between non-cryopreserved cells (A, D, G, J) and cells cryopreserved for 14 (B, E, H, K) and 202.7 ± 13.0 (C, F, I, L) days. The cultures were counterstained with a vectashield medium with DAPI (blue) and phalloidin-FITC (green) or phalloidin-TRITC (red). Scale bars, 100 μ m.

Conjunctival epithelial cells were also serially cultured until P₄ and the expression of putative progenitor cell markers p63 α and ABCG2 were followed over the passages. As the morphology of the cells changed to a larger and more flattened shape during the culture period the expression of p63 α decreased considerably (Fig. 13A-D), as cells with a more differentiated morphology were less brightly stained or negative (Fig. 13C+D, Arrowheads). However, some small cuboidal cells with p63 α positivity were observed until P₄ (Fig. 13D, Arrow). The expression of ABCG2 was stronger in cells of small size with a high n/c ratio (Fig. 13E, Arrowhead) at P₁, but at later passages cells with a more differentiated morphology remained positive for ABCG2 (Fig. 13F+H, Arrowhead). The extent of ABCG2 positive cells decreased over the culture period, but ABCG2 positive cells were still noted at P₄. No difference in expression of p63 α and ABCG2 was noted between non-cryopreserved cells and cells cryopreserved for 14 days during serial passaging.

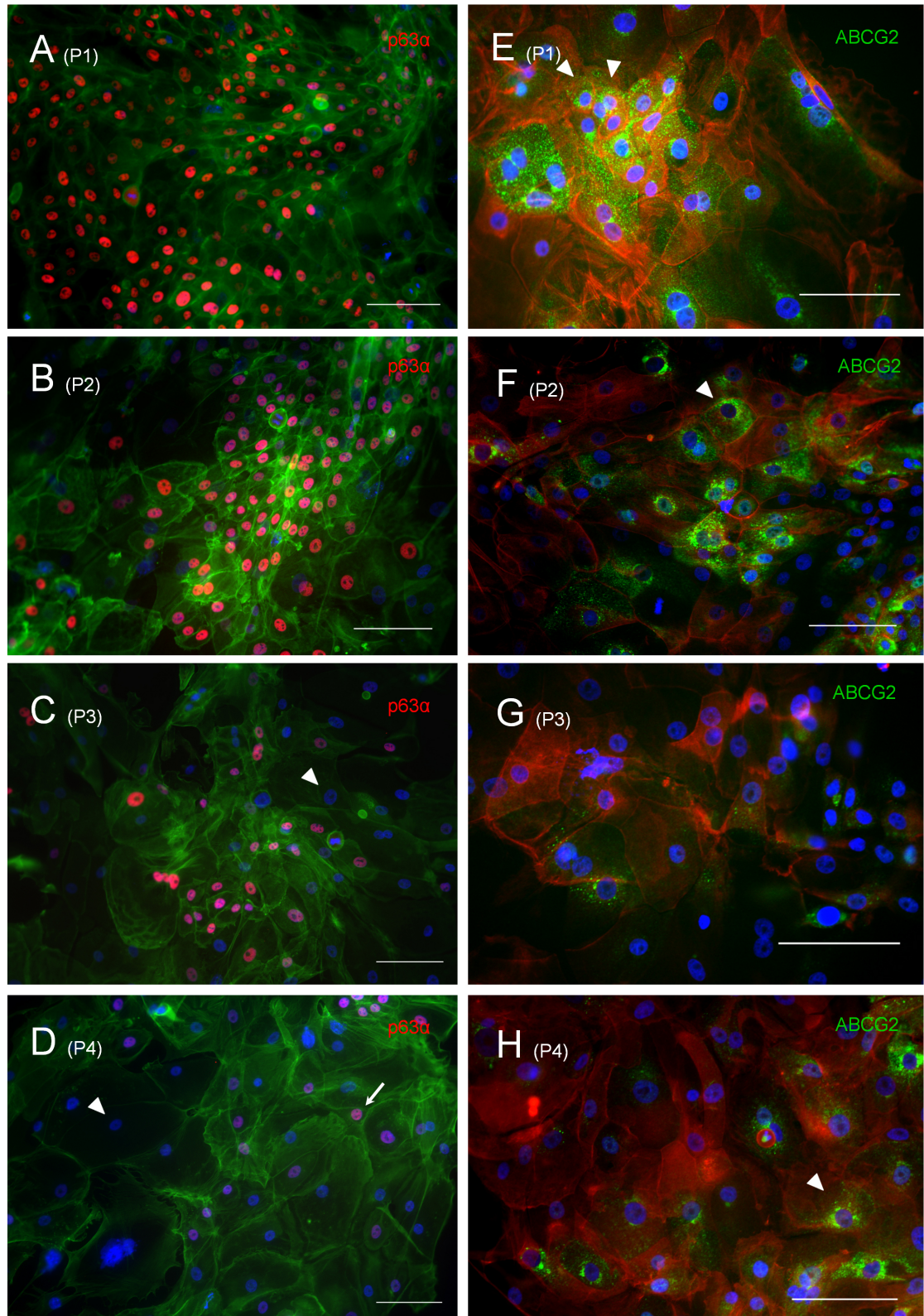


Figure 13: Putative progenitor cell marker expression during serial expansion at P₁ (A, E), P₂ (B, F), P₃ (C, G) and P₄ (D, H) for p63α (A-D) and ABCG2 (E-H).

Immunoreactivity for p63 α (red) decreased over the culture period as it was less expressed by larger, flattened cells (C+D, arrowheads), but small cuboidal cells with p63 α positivity were found until P₄ (D, Arrow). ABCG2 (green) seemed to be more strongly expressed in cells with a high n/c ratio (E, arrowheads), but also some cells with a differentiated morphology were positive for ABCG2 (F+H, arrowheads). The cultures were counterstained with a vectashield medium with DAPI (blue) and phalloidin-FITC (green) or phalloidin-TRITC (red). Scale bars, 100 μ m.

Among the six existing p63 isoforms, the Δ Np63 α isoform has shown to be most dominant in epithelial cells with stem cell like characteristics (Kawasaki, Tanioka et al. 2006). As a specific antibody against the Δ Np63 α isoform was not commercially available at the time of the study, we also performed semi-quantitative RT-PCR with specific primers for the Δ Np63 α isoform. These results of the RT-PCR confirmed Δ Np63 α and ABCG2 expression in non-cryopreserved and cryopreserved conjunctival epithelial cells until P₃. Due to a lack of cell proliferation at P₄ it was only possible to isolate enough RNA at P₄ from one donor, which also showed Δ Np63 α and ABCG2 expression before and after cryopreservation (Fig. 14).

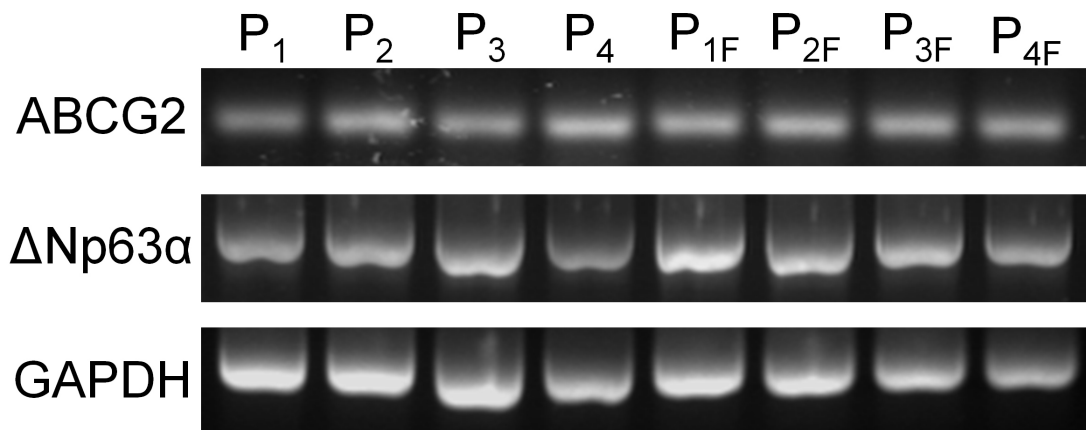


Figure 14: Results of RT-PCR analysis from P₁-P₄.

Both non-cryopreserved conjunctival epithelial cells (P₁-P₄) and conjunctival epithelial cells cryopreserved for 14 days (P_{1F}-P_{4F}) showed ABCG2 and ΔNp63α expression until passage 4.

4.3.3. Cell viability and colony forming efficiency

The cell viability of the conjunctival epithelial cells was 79.8 % ± 10.5 % after 14 days and 82.0 % ± 6.8 % after 202.7 ± 13.0 days of cryopreservation. No significant difference in cell viability was found between the two time points (Fig. 15A). The colony forming efficiency data showed no significant difference in the total colony forming capacity or in the phenotype of large colonies with smooth perimeters and small tightly packed cells between non-cryopreserved cells and cells cryopreserved for 14 and 202.7 ± 13.0 days at P₀ (Fig. 15B).

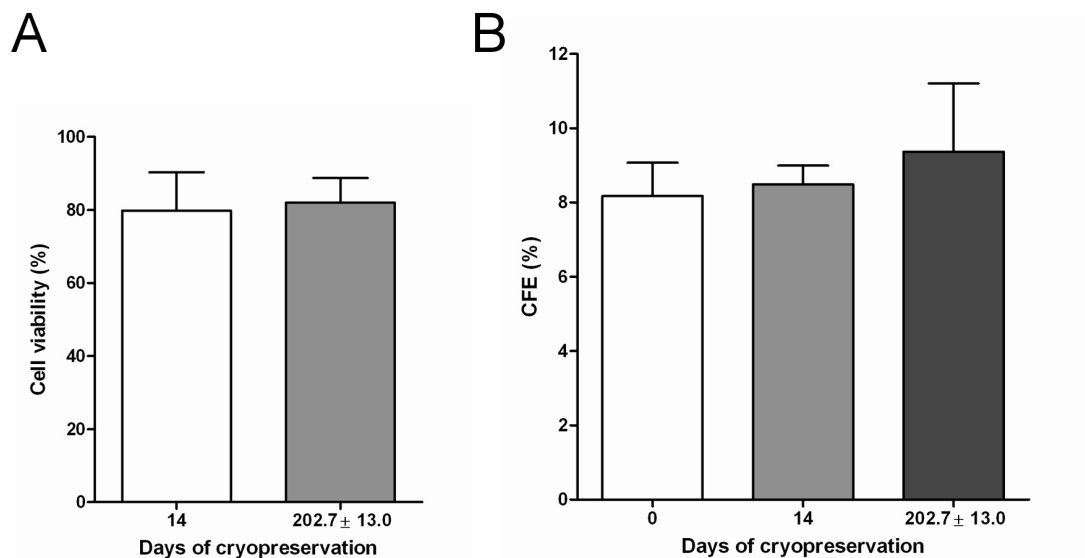


Figure 15: Cell viability and colony forming efficiency.

The cell viability of the conjunctival epithelial cells was 79.8 % ± 10.5 % after 14 days and 82.0 % ± 6.8 % after 202.7 ± 13.0 days of cryopreservation. No significant difference in cell viability was found between the two time points (A). The colony forming efficiency assays from P₀ showed no significant difference in the total colony forming capacity between non-cryopreserved cells and cells cryopreserved for 14 and 202.7 ± 13.0 days (B). All error bars represent standard deviation of the mean.

4.3.4. Colony forming efficiency and cell doubling rate during serial cultivation

During the serial cultivation of non-cryopreserved cells and cells cryopreserved for 14 days, the analysis of variance (One-Way ANOVA-Test) showed a significant difference between each of the four passages ($p \leq 0.05$). A

significant reduction in colony forming efficiency was observed between P_0 and P_1 and P_1 and P_2 in both non-cryopreserved and cryopreserved conjunctival epithelial cells (Fig. 16). Conjunctival epithelial cells from non-cryopreserved and cryopreserved samples usually reached senescence at P_4 . Calculation of the cell population doubling rates showed that non-cryopreserved and cryopreserved cells underwent 19.06 ± 0.43 and 20.58 ± 1.34 cell doublings respectively before senescence. No significant difference was found between non-cryopreserved and cryopreserved cells (Fig. 16).

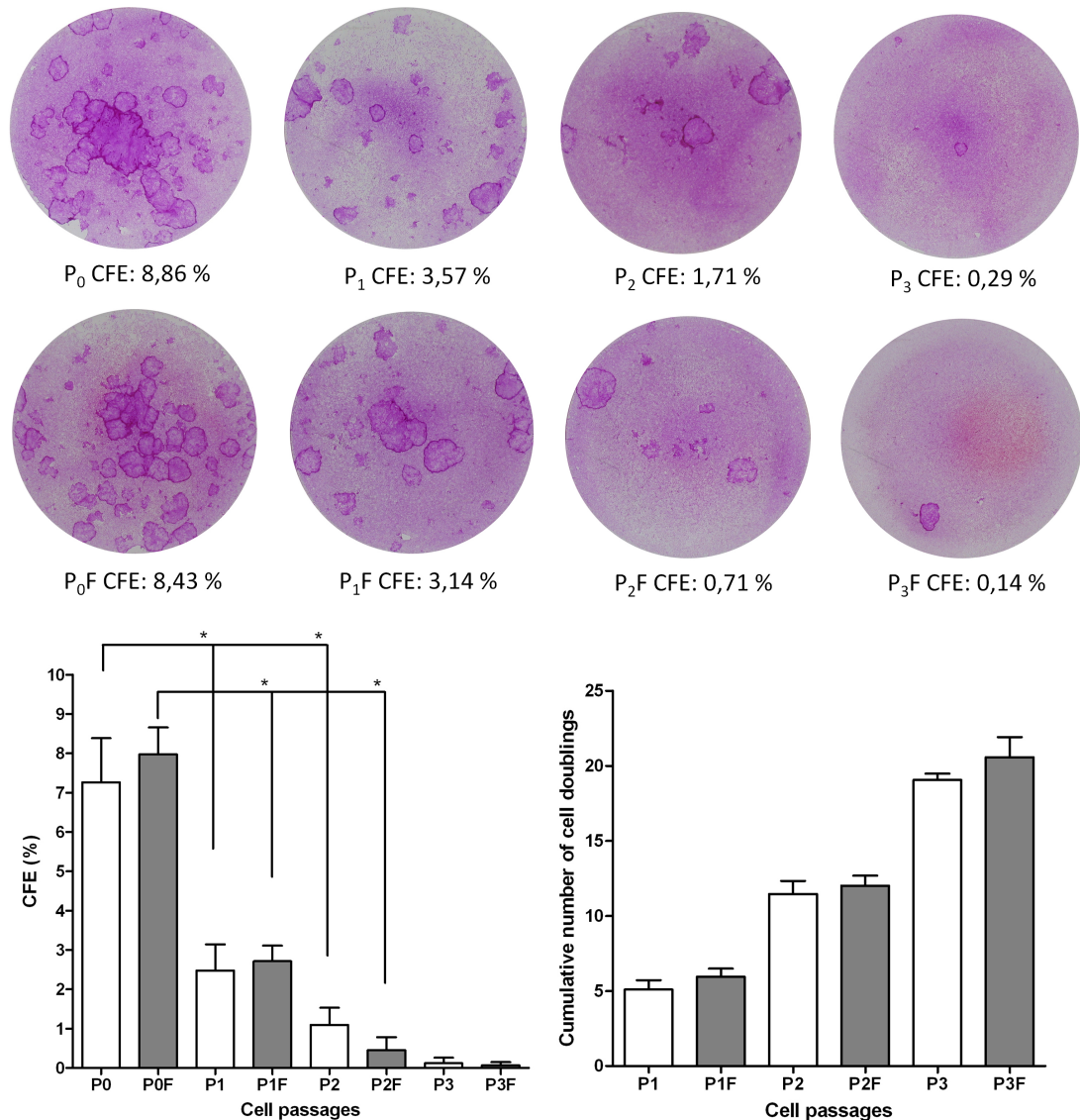


Figure 16: Colony forming efficiency and cumulative cell doubling rate from non-cryopreserved conjunctival epithelial cells (P₀-P₃) and conjunctival epithelial cells cryopreserved for 14 days (P₀F-P₃F).

The colony forming efficiency data showed no significant difference in the total colony forming capacity or in the phenotype of large colonies with smooth perimeters and small tightly packed cells between non-cryopreserved and cryopreserved cells at P₀. A significant reduction in colony forming efficiency was observed between P₀ and P₁ and P₁ and P₂ in non-cryopreserved and cryopreserved conjunctival epithelial cells. The cumulative cell doubling rate for non-cryopreserved (P₁-P₃) and cryopreserved cells (P₁F-P₃F), showed that non-cryopreserved and

cryopreserved cells underwent 19.06 ± 0.43 and 20.58 ± 1.34 cell doublings respectively before senescence. All error bars represent standard deviation of the mean.

4.4. Discussion

A major problem for the clinical application of cell based therapies, such as the transplantation of epithelialised cell sheets, is the shortage of donor tissue and ensuring an epithelialised graft is available when a patient needs it. The use of cryopreserved conjunctival epithelial cells could significantly facilitate the delivery of grafts from the laboratory to the clinic. As cells with stem cell characteristics are needed to ensure long-term epithelial function in-vivo after transplantation (Pellegrini, Golisano et al. 1999; Henderson and Collin 2008), we investigated the effect of cryopreservation and long-term culture on the progenitor cell characteristics and proliferative capacity of conjunctival epithelial cells.

Freezing injury of cells during cryopreservation has shown to have two components: direct damage from ice crystals and a secondary damage caused by the increase in concentration of solute as a result of gradual ice formation (Karlsson, Cravalho et al. 1993). Many chemicals have been found to have cryoprotective properties, and among these, Dimethyl sulfoxide (DMSO) and glycerol are the ones currently most commonly used (Elmoazzen, Elliott et al. 2005; Keros, Rosenlund et al. 2005). According to their ability to enter the cell, cryoprotectants are classified into two groups: permeating cryoprotectants, including glycerol, DMSO and ethylene glycol, and nonpermeating

cryoprotectants, including hydroxyethyl starch and various carbohydrates (Oh, Kim et al. 2007). Although the mechanism underlying cryoprotection by these components is not fully understood, both permeating and non permeating cryoprotectants reduce the concentration of intracellular water, resulting in a reduction in the rate at which the water molecules can form potentially harmful ice crystals (Karlsson, Cravalho et al. 1993). In addition to the use of these cryoprotectants, serum supplementation has shown to be beneficial to the ability of cells to survive cryopreservation in a variety of cells (Son, Kim et al. 2004; Men, Agca et al. 2005; Disis, dela Rosa et al. 2006).

A variety of cryopreservation protocols have been developed, however the optimal protocol may differ depending on cell type and species of origin (Armitage 1987). DMSO was chosen for this study as it is one of the most widely used cryoprotectants for cells (Kito, Kagami et al. 2005). It has also been used to cryoprotect corneal grafts, which have been successfully applied in many clinical cases (Taylor and Hunt 1989). DMSO has also been shown to be superior to glycerol for the cryopreservation of human limbal epithelial stem cells on amniotic membrane (Yeh, Yao et al. 2008). In our study, the cell viability after was 79.8 ± 10.5 % after 14 days and 82.0 ± 6.8 % after 202.7 ± 13.0 days of cryopreservation, which is comparable to the cell viability found by others for human limbal epithelial stem cells (79.8 ± 4.01 %) and slightly less than for rabbit conjunctival epithelial cells (89.0 ± 6.14 %) cryopreserved under similar conditions (Oh, Kim et al. 2007). The characterisation of the conjunctival epithelial cells showed strong positivity for CK19, whereas CK4 immunoreactivity was mainly found in large flattened differentiated cells. This

is in accordance with the findings by Risse-Marsh et al. who also observed intense CK4 reactivity in superficial and suprabasal conjunctival cells, whereas the great majority of basal cells showed no detectable staining for CK4 (Risse Marsh, Massaro-Giordano et al. 2002). The conjunctival cells showed strong expression of p63 α at P₁, which decreased over the culture period, but was still detectable at P₄. Expression of p63 α was localised to small cuboidal cells with a high n/c ratio and expression decreased when cells became more flattened and differentiated over the culture period. Expression of the ABCG2 transporter protein was also observed in the cytoplasm of the conjunctival epithelial cells until P₄. The expression decreased over the culture period and seemed to be stronger in the small cuboidal cells, but also remained in the flattened differentiated cells. No difference in CK19, CK4, p63 α and ABCG2 expression was noted between non-cryopreserved and cryopreserved cells. In a study by Yeh et al. comparable immunoreactivity to ABCG2 of limbal epithelial stem cells cultivated on amniotic membrane before and after cryopreservation was noted (Yeh, Yao et al. 2008). The transcription factor p63 α and the transporter protein ABCG2 are putative epithelial progenitor cell markers (Pellegrini, Dellambra et al. 2001; Watanabe, Nishida et al. 2004; Budak, Alpdogan et al. 2005; Kawasaki, Tanioka et al. 2006), which have also been detected in the basal cell layer of the conjunctiva (Tanioka, Kawasaki et al. 2006). The decrease of p63 α and ABCG2 immunoreactivity over the culture period in our study indicates a gradual loss of progenitor cells which also correlated with a decrease in the number of cells able to found colonies with smooth perimeters containing small tightly packed cells over the culture period.

Evaluation of the proliferative potential showed that non-cryopreserved and cryopreserved cells underwent 19.06 ± 0.43 and 20.58 ± 1.34 cell doublings respectively before senescence. These results are in accordance with observations of the proliferative capacity of epidermal keratinocytes, which showed cell doubling rates of 20-27 cell generations before senescence in donors aged 3-34 years (Rheinwald and Green 1975). Cryopreservation seems to have no effect on the colony forming efficiency and the proliferative potential of the cells, as no significant difference was found in these parameters before and after cryopreservation. Additionally, a cryopreservation duration of more than 6 months seems not to alter the progenitor cell characteristics of conjunctival epithelial cells as no difference was observed in cell viability, immunoreactivity to p63 α and ABCG2 and colony forming capacity between 14 days and 202.7 ± 13.0 days of cryopreservation. However it is possible that the cryopreservation process affects other parameters, like the clonal analysis or goblet cell differentiation which was not investigated in this study.

In conclusion cryopreservation with CECM supplemented with 10% DMSO and 20% FBS was effective in preserving conjunctival epithelial cells with progenitor cell characteristics and it had no detectable effect on the progenitor cell marker expression, the colony forming efficiency and the proliferative capacity during long term in-vitro expansion. The use of cryopreserved conjunctival epithelial cells could improve the supply of tissue for cell therapy applications in the clinic.

Chapter 5:

Simulation of an environment *in vitro* which is more similar to the stem cell niche in vivo in order to preserve conjunctival epithelial progenitor cells during cell expansion *in vitro*

5. Simulation of an environment *in vitro* which is more similar to the stem cell niche *in vivo* in order to preserve conjunctival epithelial progenitor cells during cell expansion *in vitro*

5.1. Introduction

In vivo, survival, proliferation and differentiation of epithelia depend on a complex system of interactions between epithelial stem cells, neighbouring cells, the local environment and the underlying mesenchymal stroma, which together comprise the stem cell “niche” (Spradling, Drummond-Barbosa et al. 2001; Blanpain, Horsley et al. 2007; Revoltella, Papini et al. 2007). Stem cells are regulated in their “niches” by intercellular interactions, the external environment and the underlying mesenchyme (Revoltella, Papini et al. 2007) and it has been shown that these tissue specific niche environments are essential for stem cell maintenance and the control of the epithelial cell-fate determination (Spradling, Drummond-Barbosa et al. 2001; Blanpain, Horsley et al. 2007) (Fig. 17).

Major advances in the treatment of patients with ocular surface disease have been made by culturing limbal and conjunctival epithelial cells from small biopsies on substrates like amniotic membrane or fibrin and transplanting these cell-matrix constructs back to the affected ocular surface (Tsai, Li et al. 2000; Tan, Ang et al. 2004; Ang, Tan et al. 2005; Rama, Matuska et al. 2010).

Apparently, such culture systems rely on the successful expansion of the progenitor cell population *in vitro*.

Without matrix or fibroblast support, the proliferation of epithelial cells *in vitro* is extremely limited as epithelial cells lose their progenitor cell population and undergo rapid differentiation on conventional plastic or glass substrates (Tsai, Ho et al. 1994; Little, Gawkrödger et al. 1996; Sun, Higham et al. 2004; Schrader, Notara et al. 2009). The gold standard technique for the culture of epithelial cells includes the use of foetal calf serum and growth arrested murine 3T3 feeder cells (Rheinwald and Green 1975; Pellegrini, Golisano et al. 1999; Ang, Tan et al. 2004). However, the use of serum and murine cells in the *ex vivo* expansion of epithelial cells for clinical transplantation not only poses the risk of transmission of various adventitious agents, but furthermore as described in the previous chapter, even using this gold standard technique results in a considerable decrease of cells with stem cell characteristics during epithelial cell expansion *in vitro*.

Tissue specific stromal fibroblasts are likely to play an important role in the regulation of progenitor epithelial cell differentiation both *in vivo* and *in vitro*, by providing growth factors and cytokines that could mediate complex paracrine interactions between epithelial cells and fibroblasts (Li and Tseng 1995; Spradling, Drummond-Barbosa et al. 2001).

Therefore, based on previous work on epidermal epithelial cells by Sheila Mac Neil (Sun, Higham et al. 2004) the aim of this experimental series was to evaluate a serum free co-culture system where mitotically active human conjunctival fibroblasts were cultured together with conjunctival epithelial cells

to mimic an environment *in vitro*, which is more similar to the stem cell niche *in vivo* in order to preserve the conjunctival epithelial progenitor cell population during *in vitro* expansion.

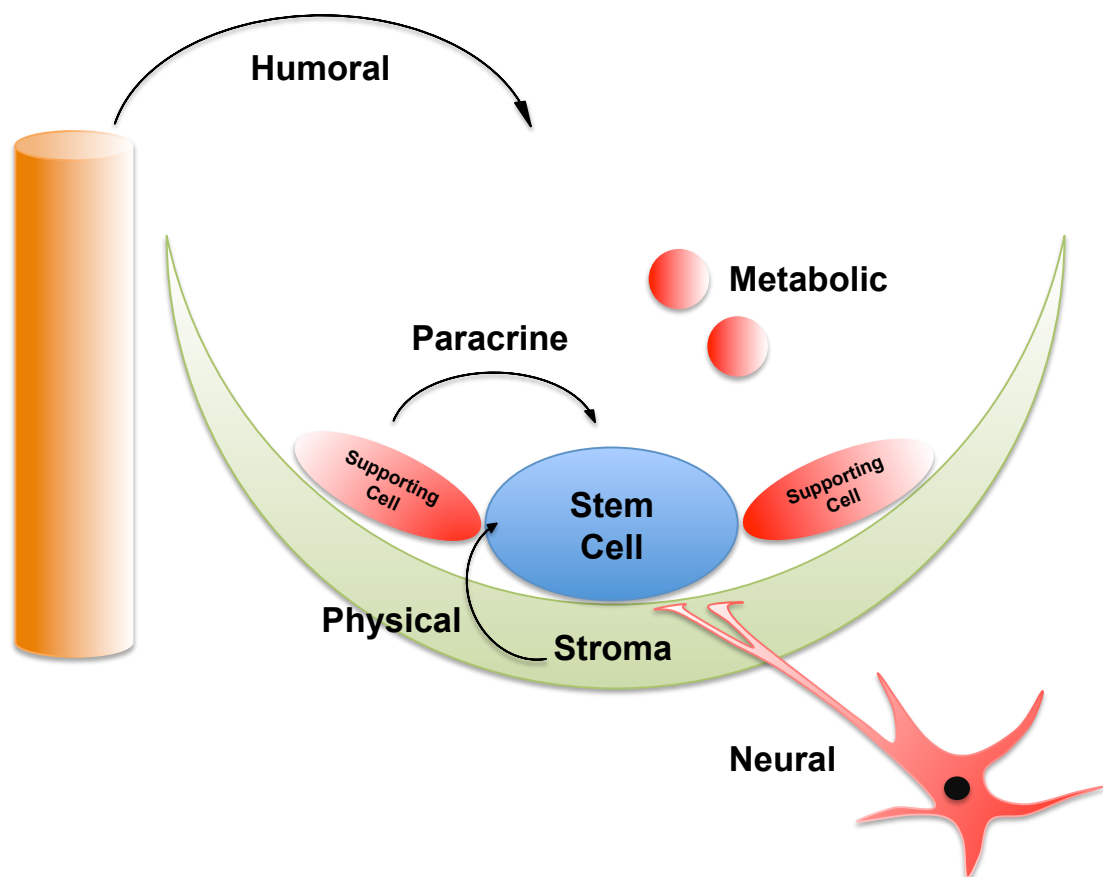


Figure 17: Interactions in the stem cell niche.

In vivo, survival, proliferation and differentiation of epithelia depend on a complex system of interactions between epithelial stem cells, neighbouring cells, the local environment and the underlying mesenchymal stroma, which together comprise the stem cell “niche”. These tissue specific niche environments are essential for stem cell maintenance and the control of the epithelial cell-fate determination. Adapted from Scadden et al. (Scadden 2006).

5.2. Methods

The methods used for the experiments in this chapter are described in chapter 2 (Materials and Methods). Conjunctival cells from eight conjunctival biopsies from eight different donors were used for the experiments with three repeats for each set of experiments and three repetitions per experiment. The methods specific to this chapter are described below.

5.2.1. Human conjunctival fibroblast Isolation and culture

Human conjunctival fibroblasts were grown out of approximately 2x2 mm conjunctival tissue explants from which the epithelium had been removed after 2 hours treatment using Dispase II (2mg/ml) (Roche Diagnostics). The fibroblasts were cultured in DMEM (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (Gibco). For the setup of the co-cultures, human conjunctival fibroblasts not older than passage six, were used.

5.2.2. Setup of the conjunctival epithelial cell/conjunctival fibroblast co-cultures

The co-cultures were established using primary human conjunctival epithelial cells and human bulbar conjunctival fibroblasts (HCEC-HCF) and were followed up to 6 days when they reached near or total confluence. Conjunctival epithelial cells grown on growth arrested 3T3 feeder cells were used as a control (HCEC-3T3). For the co-culture system conjunctival epithelial cells

were seeded at a density of 2.4×10^4 cells/cm² and the conjunctival fibroblasts at a density of 0.8×10^4 cells/cm² (ratio 3:1). For the 3T3-cultures the same conjunctival epithelial cell density as in the co-culture system was seeded (2.4×10^4 cells/cm²). The HCEC-3T3 cultures were grown in CECM and the HCEC-HCF cultures were grown in CECM omitting the serum or any other serum replacement (Fig. 18).

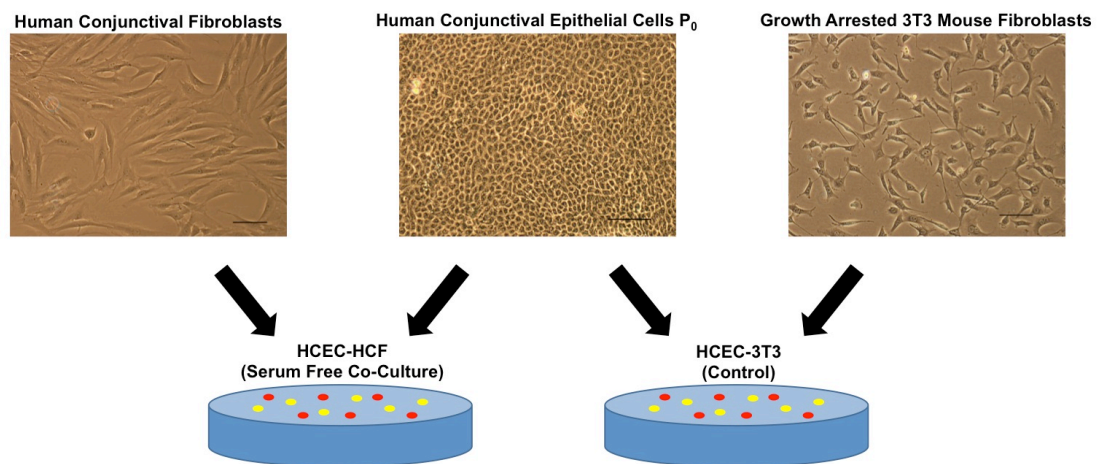


Figure 18: Experimental setup of the HCEC-HCF co-cultures and the HCEC-3T3 control group.

5.2.3. Two step trypsinisation method

To ensure pure epithelial cell samples were obtained from both groups for the analysis, a two step trypsinisation method was used. Trypsin (0.05%) with 0.2g/L EDTA (1x trypsin with EDTA) was firstly used to selectively remove the fibroblasts, followed by 10x trypsin with EDTA to obtain the conjunctival

epithelial cells. The two step trypsinisation method has been validated using specific antibodies against the conjunctival epithelial cell marker CK19 and the epithelial progenitor cell marker p63 α . The results showed that under HCEC-3T3 and HCEC-HCF conditions, the first step of the trypsinisation successfully removed the CK19 and p63 α negative 3T3 feeder cells and human conjunctival fibroblasts, while the CK19 and p63 α positive conjunctival epithelial cells remained attached to the culture plate and were then isolated during second trypsinisation step (Fig. 19).

1. Two Step Trypsinisation

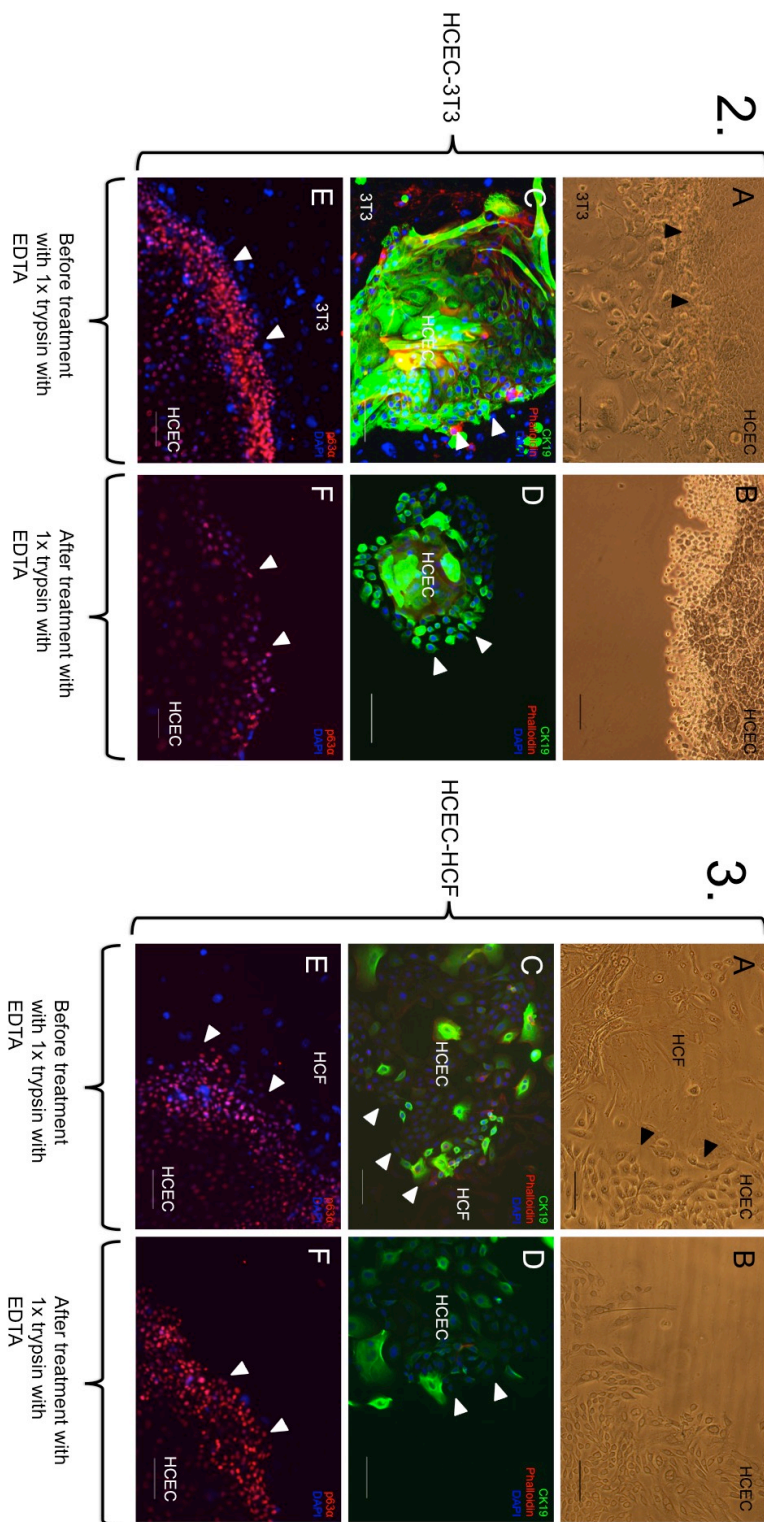
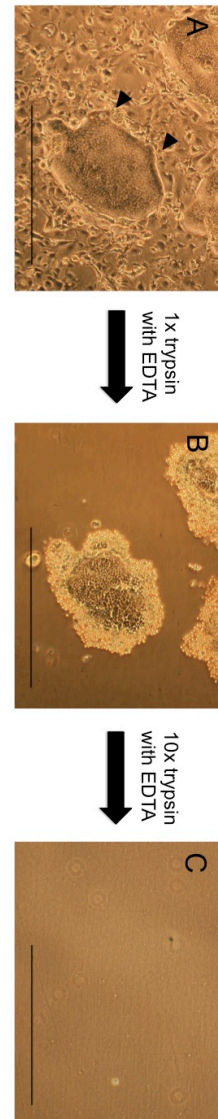


Figure 19: Two step trypsinisation method.

1. A-C: Principle of the two step trypsinisation, showing successful removal of the fibroblasts after the first trypsinisation step (1x trypsin with EDTA), while the epithelial cells remain attached to the culture plate

(B) and removal of the epithelial cells after the second trypsinisation step (10x trypsin with EDTA) (C). 2. + 3.: Conjunctival epithelial cells cultured under HCEC-3T3 (2. A-F) and HCEC-HCF (3. A-F) conditions before and after the first trypsinisation step. Under both conditions the first step of the trypsinisation successfully removed the CK19 (green, C+D) and p63 α (red, E+F) negative 3T3 feeder cells (2.) and human conjunctival fibroblasts (3.), while the CK19 (C+D) and p63 α (E+F) positive conjunctival epithelial cell colonies (arrowheads) remained attached to the culture plate. The cultures were counterstained with a vectashield medium with DAPI (blue). Scale bars: 1. A-C: 1 mm, 2. + 3. A-F: 100 μ m.

5.2.4. Comparison of the cell proliferation dynamics in the HCEC-HCF co-culture system under serum free and serum containing conditions

For the comparison of the cell proliferation dynamics between the conjunctival epithelial cells and the conjunctival fibroblasts in the HCEC-HCF co-culture system under serum free and serum containing conditions the cells were cultured in CECM with and without foetal calf serum. The cell proliferation was compared by immunocytochemistry using an antibody against the KI67 protein, a marker of cell proliferation, which is present during all active phases of the cell cycle (G_1 , S, G_2 and mitosis), but absent in resting cells (G_0) (Scholzen and Gerdes 2000)

5.3. Results

5.3.1. Proliferation of conjunctival epithelial cells in the HCEC-HCF system using different epithelial cell : fibroblast ratios

The proliferation dynamics between epithelial cells and non growth arrested fibroblasts under serum free conditions have already been investigated by Sun et al. (Sun, Higham et al. 2004). Our Initial experiments confirmed their results for conjunctival epithelial cells, by showing that conjunctival epithelial cells cultured under HCEC-HCF conditions with a seeding density of 3:1 (2.4×10^4 cells/cm² epithelial cells and 0.8×10^4 cells/cm² fibroblasts) in serum free CECM consistently showed strong epithelial cell proliferation and approximately 90% epithelial cell confluence after 1 week of culture (Fig. 20D-F). We also found, that a higher seeding ratio of 2:1 (2.4×10^4 cells/cm² epithelial cells and 1.2×10^4 cells/cm² fibroblasts) regularly resulted in less epithelial cell confluence with fibroblasts remaining after 1 week of co-culture (Fig 20A-C). When the number of seeded fibroblasts was reduced to a 5:1 ratio (2.4×10^4 cells/cm² epithelial cells and 0.48×10^4 cells/cm² fibroblasts), also epithelial cells did not reach confluence after one week of culture (Fig. 20G-I). These observations are in accordance with the results of Sun et al., who found, that a minimum seeding density of fibroblasts is needed for successful epithelial cell expansion, but that a too high ratio of fibroblasts to epithelial cells also limits epithelial cell growth (Sun, Higham et al. 2004).

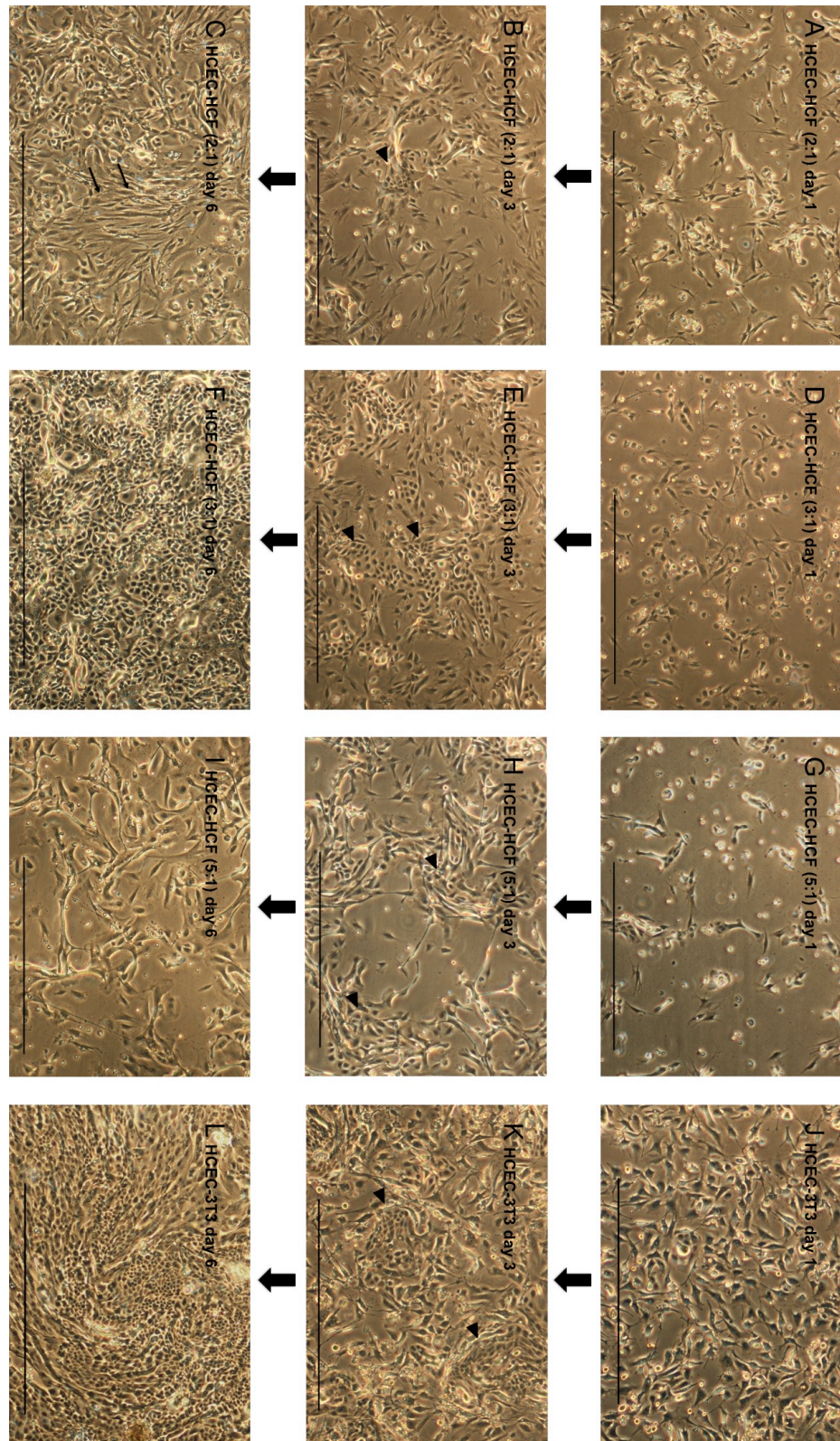


Figure 20: Proliferation of conjunctival epithelial cells in the HCEC-HCF co-culture system at day 1, 3, 6, using different seeding ratios of epithelial cells and fibroblasts.

All tested seeding ratios resulted in formation of epithelial cell colonies after 3 days of culture (arrowheads). However, high numbers of fibroblasts (seeding ratio of 2:1) regularly resulted in less epithelial cell confluence with remaining fibroblasts in the culture (arrows) after 1 week of co-culture (A-C). However, seeding ratios of 3:1, consistently showed strong epithelial cell proliferation and approximately 90% epithelial cell confluence after 1 week of culture (D-F). When the number of seeded fibroblasts was further reduced (5:1 ratio), epithelial cells also showed less proliferation and did not reach confluence after one week of culture (18G-I). J-L: Control condition, of conjunctival epithelial cells cultured on a growth arrested 3T3 feeder layer (HCEC-3T3). The cells showed good proliferation and confluence after approximately 1 week in culture. Scale bars, 1 mm.

5.3.2. Cell growth and morphology

After 1 day of culture single conjunctival epithelial cells and small epithelial cell groups were found in the HCEC-HCF culture-system (Fig. 21A, Arrowheads). The epithelial cells were surrounded and in direct contact with the subconjunctival fibroblasts (Fig. 21A, Arrow). On day 3 larger epithelial cell colonies were observed under HCEC-HCF conditions (Fig. 21B, Arrowheads), which were still surrounded by supportive subconjunctival fibroblasts (Fig. 21B, Arrow). At day 6 a subconfluent monolayer containing a majority of small cuboidal conjunctival epithelial cells with a high nuclear to cytoplasmatic (n/c) ratio was found (Fig. 21C). In the HCEC-3T3 system growing epithelial cell colonies were also found during the culture period (Fig. 21D+E, Arrowheads). At day 6 the epithelial cells had also reached subconfluence, however, the nature of the cell population was more heterogenous and alongside epithelial

cells with a high n/c ratio, areas containing differentiated epithelial cells with a squamous shape and a lower n/c ratio were frequently noted (Fig. 21F, Arrowheads).

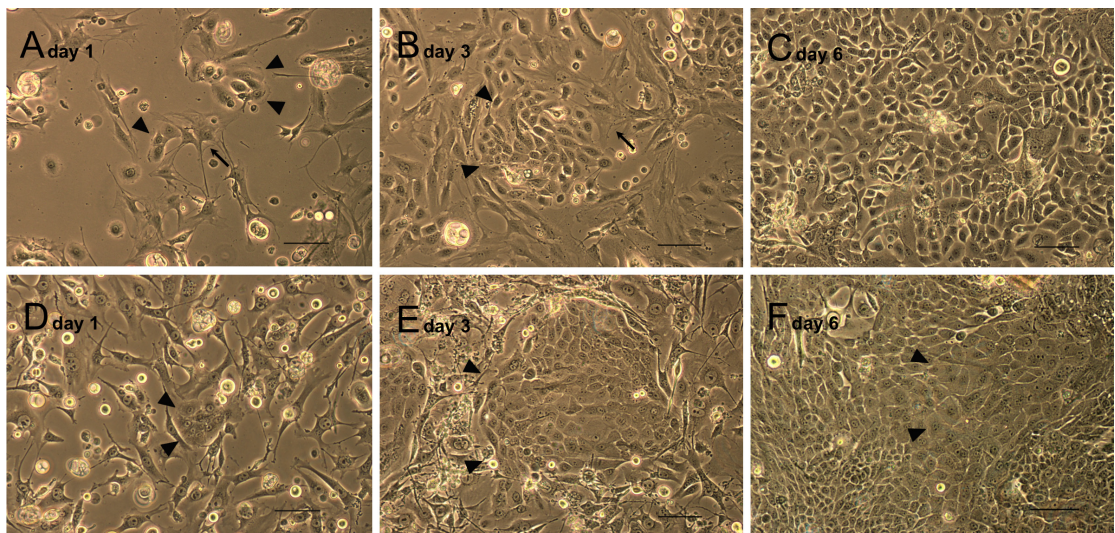


Figure 21: Cell growth under HCEC-HCF (A-C) and HCEC-3T3 (D-F) conditions.

A: Single conjunctival epithelial cells and small epithelial cell groups were found in the HCEC-HCF culture-system after 1 day of culture (Arrowheads). The epithelial cells are surrounded and in direct contact with the conjunctival fibroblasts (Arrow). **B:** On day 3 larger epithelial cell colonies were observed (Arrowheads), which were still surrounded by supportive conjunctival fibroblasts (Arrow). **C:** At day 6 a subconfluent monolayer containing a majority of small cuboidal conjunctival epithelial cells with a high nuclear to cytoplasmatic (n/c) ratio was found. **D+E:** In the HCEC-3T3 system growing epithelial cell colonies were also found during the culture period (Arrowheads). At day 6 the epithelial cells had also reached subconfluence, however, the nature of the cell population was more heterogenous and alongside epithelial cells with a high n/c ratio, areas containing differentiated epithelial cells with a squamous shape and a lower n/c ratio were frequently noted (F, Arrowheads). Scale bars, 100 μ m.

5.3.3. Cell Proliferation in the HCEC-HCF Co-culture System

Immunostaining for the proliferative marker Ki67 showed that under serum free conditions some of the conjunctival epithelial cells (Fig. 22A, arrowhead) and some of the subconjunctival fibroblasts (Fig. 22A, arrow) expressed Ki67 in the HCEC-HCF co-culture system at day 1, indicating that the two cell populations were increasing in number. However, after 3 days of culture an increasing number of Ki67 positive epithelial cells were noted (Fig. 22B, arrowheads), surrounded mainly by Ki67 negative fibroblasts (Fig. 22B, arrows). At day 6 the cultures were dominated by confluent small tightly packed Ki67 positive epithelial cells (Fig. 22C). The converse was found when the same cell ratios were seeded and then cultured in the presence of serum. A large number of fibroblasts expressing Ki67 were already found at day 1 (Fig. 22D, arrows). The fibroblasts started to overgrow the culture at around day 3 (Fig. 22E), leaving only small islands of differentiated, flattened shaped epithelial cells surrounded by proliferating fibroblasts at day 6 (Fig. 22F). This suggests that serum changes the balance of the system for the benefit of the fibroblasts thus masking potentially important supporting interactions between the two cell populations.

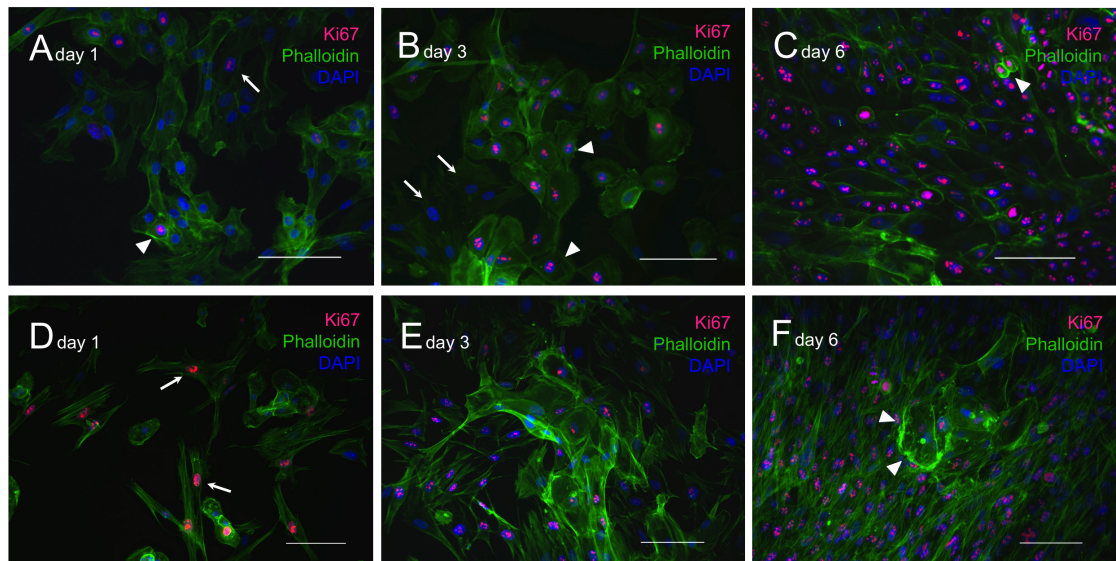


Figure 22: Expression of Ki67 in the HCEC-HCF co-culture system under serum free conditions and in the presence of serum.

A: Under serum free conditions some conjunctival epithelial cells (Arrowhead) and some subconjunctival fibroblasts (arrow) expressed Ki67 (red) at day 1. **B:** After 3 days of culture an increasing number of Ki67 positive epithelial cells were noted (Arrowheads), surrounded mainly by Ki67 negative fibroblasts (arrows). **C:** At day 6 the cultures were dominated by confluent small tightly packed Ki67 positive epithelial cells (arrowhead: Cell division). **D:** In the presence of serum, a large number of fibroblasts expressing Ki67 were found at day 1 (arrows). **E+F:** The fibroblasts started to overgrow the culture at around day 3, leaving only small islands of differentiated, flattened shaped epithelial cells surrounded by proliferating fibroblasts at day 6 (F, arrowheads). The cultures were counterstained with a vectashield medium with DAPI (blue) and phalloidin-FITC (green, A-F). Scale bars, 100 μ m.

5.3.4. Expression of Progenitor Cell Markers

Conjunctival epithelial cells cultured under HCEC-HCF and HCEC-3T3 conditions showed strong immunoreactivity to the putative progenitor cell

markers p63 α and ABCG2 (Fig. 23A+B and D+E) and these results were confirmed by RT-PCR (Fig. 23G). Co-localisation of cytokeratin 15 (CK15) and p63 α revealed a subpopulation of CK15 positive cells inside the population of p63 α positive cells in the HCEC-3T3 group (Fig. 23F), whereas under HCEC-HCF conditions significantly fewer CK15 positive cells were found (Fig. 23C, Arrowheads + Fig. 23H). These results were in accordance with the RT-PCR data, which showed less expression of CK15 in the HCEC-HCF group (Fig. 23G).

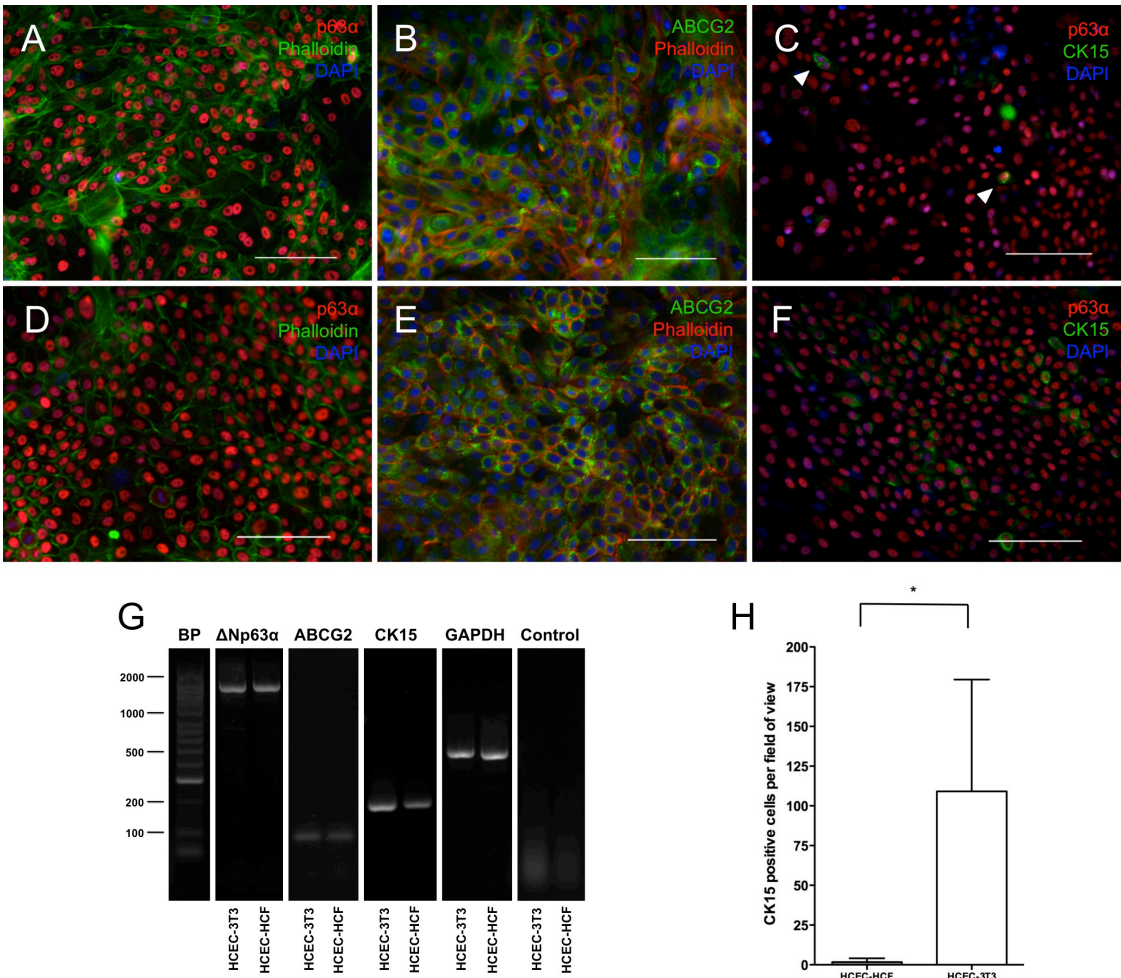


Figure 23: Expression of putative progenitor cell markers under HCEC-HCF (A-C) and HCEC-3T3 (D-F) conditions.

Conjunctival epithelial cells cultured under both conditions showed strong immunoreactivity to the putative progenitor cell markers p63 α (red, A+D) and ABCG2 (green, B+E) and these results were confirmed by RT-PCR (G). Co-localisation of CK15 (green) and p63 α (red) revealed a subpopulation of CK15 positive cells in the HCEC-3T3 group (F), whereas under HCEC-HCF conditions only few CK15 positive cells were found (C, Arrowheads). These results were in accordance with the RT-PCR, which showed less expression of CK15 in the HCEC-HCF group (G, BP=base pairs, control="no-RT" control). Also the comparison of the number of CK15 positive cells per field of view, showed significantly less CK15 positive cells per field of view in the HCEC-HCF group compared to the HCEC-3T3 group. The cultures were counterstained with a vectashield medium with DAPI (blue) and phalloidin-FITC (green, A+D) or phalloidin-TRITC (red, B+E). Scale bars, 100 μ m.

However, when single cell suspensions from conjunctival epithelial cells were transferred from the HCEC-HCF and the HCEC-3T3 co-culture system to a second dish and cultured in serum containing CECM in the presence of 3T3 feeders, colonies generated from single cells from both groups showed strong ABCG2, p63 α and CK15 positivity (Fig. 24 B+E and C+F). Small tightly packed cells with a high n/c ratio were more abundant in the periphery near the edges of the colonies in both groups, where as in the centre often squamous, differentiated cells were found (Fig. 24 A+D, Arrowheads). This was in accordance with the expression of ABCG2, p63 α and CK15, which was also stronger in cells in the periphery of the colonies (Fig. 24 B+E and C+F).

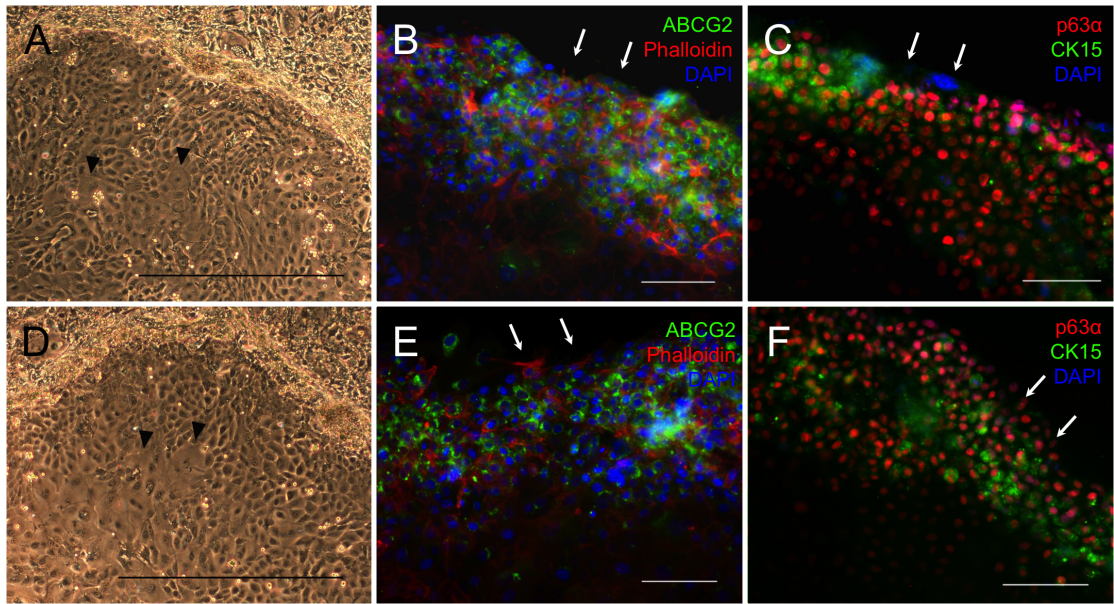


Figure 24: Conjunctival epithelial cell colonies grown in the presence of serum using cells transferred from the HCEC-HCF (A-C) and HCEC-3T3 (D-F) co-culture systems.

Small, tightly packed cells with a high n/c ratio were more abundant in the periphery near the edges of the colonies in both groups, where as in the center often squamous, differentiated cells were found (A+D, Arrowheads). The cells from both groups showed strong ABCG2 (green), p63α (red) and CK15 (green) positivity, which was more intense in cells in the periphery of the colonies (B+E and C+F). Arrows: Colony edges. The cultures were counterstained with a vectashield medium with DAPI (blue) and phalloidin-TRITC (red, B+E). Scale bars, 1mm (A+D), 100 μm (B, C, E, F).

5.3.5. Goblet Cell Differentiation

Immunostaining for the goblet cell specific mucin MUC5AC in conjunctival cells cultured under HCEC-3T3 conditions revealed the presence of small cell groups and single cells which were positive for MUC5AC (Fig. 25A + 25A inset). Also the PAS staining showed single epithelial cells and small cell

groups, which were PAS positive (Fig. 25B + 25B inset). However, in the HCEC-HCF co-culture system MUC5AC and PAS positive cells were only found very rarely (Fig. 25C + 25D). This was confirmed by RT-PCR, which showed a higher expression of MUC5AC in the HCEC-3T3 group compared to the HCEC-HCF group and also by the comparison of the number of MUC5AC positive cells per field of view, which showed significantly more MUC5AC positive cells per field of view in the HCEC-3T3 group compared to the HCEC-HCF group (Fig. 25G + 25H).

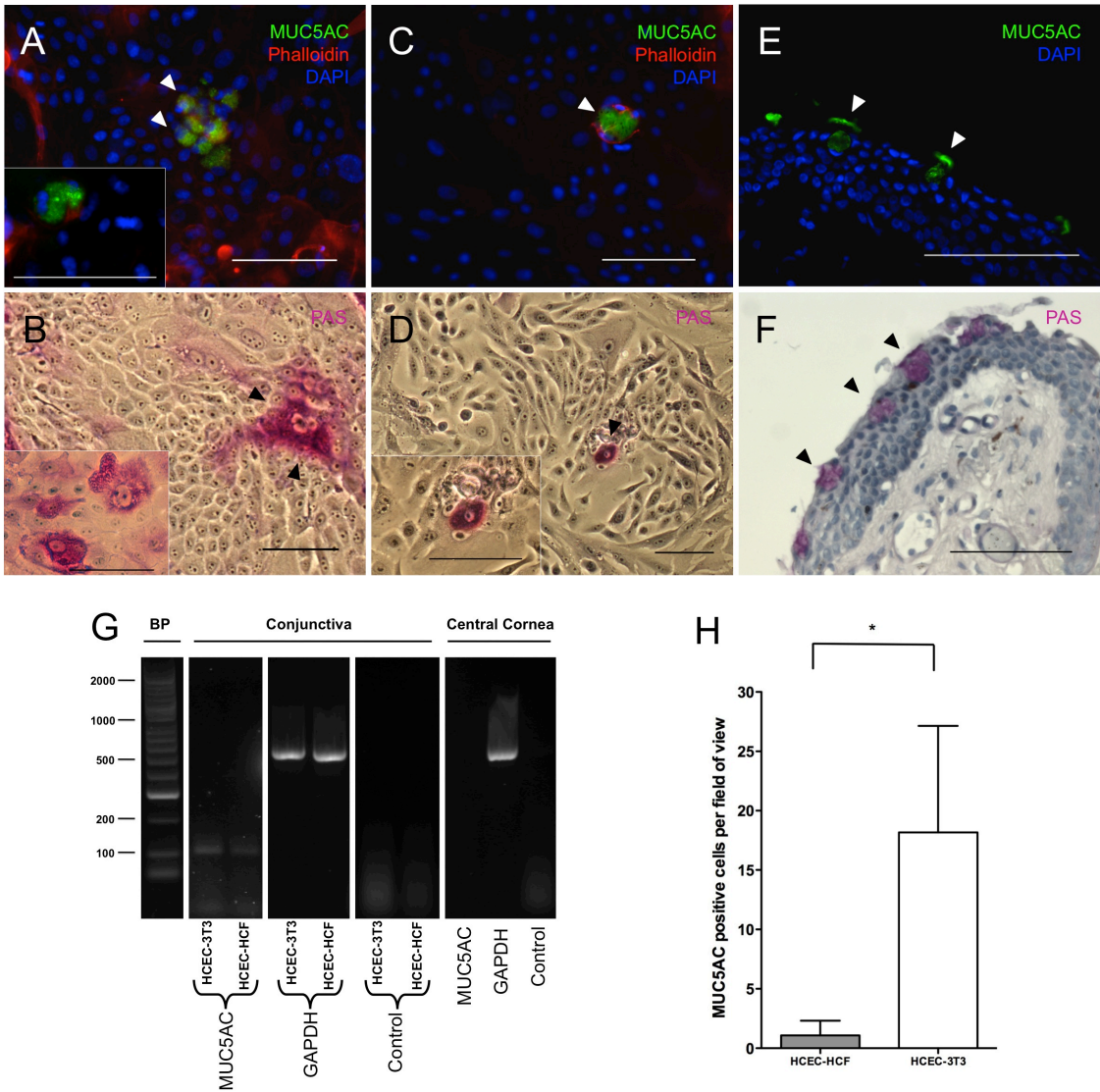


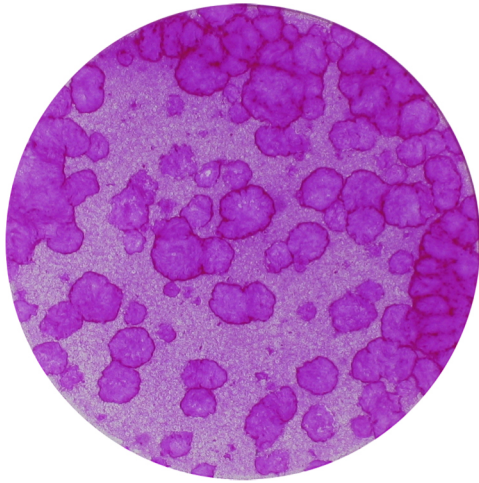
Figure 25: Expression of the goblet cell specific mucin MUC5AC and PAS-staining under HCEC-3T3 and HCEC-HCF conditions.

Under HCEC-3T3 conditions MUC5AC (green) positive single cells and small cell groups were frequently found (A + A inset). Also the PAS-staining (red) revealed single epithelial cells and small cell groups which were PAS positive (B + B inset). However, under HCEC-HCF conditions MUC5AC and PAS positive cells were only found very rarely (C+D). This was confirmed by RT-PCR which showed a higher expression of MUC5AC in the HCEC-3T3 group compared to the HCEC-HCF group (G, BP=base pairs, control="no-RT" control). Also the comparison of the number of MUC5AC positive cells per field of view, showed significantly fewer MUC5AC positive cells per field of view in the HCEC-HCF group compared to the HCEC-3T3 group (H). E+F: Conjunctival control sections showing MUC5AC positive (E, arrowheads) and PAS positive (F, arrowheads) goblet cells. The cultures were counterstained with a vectashield medium with DAPI (blue) and phalloidin-TRITC (red, A+C). Scale bars, 100 μ m.

5.3.6. Colony Forming Efficiency

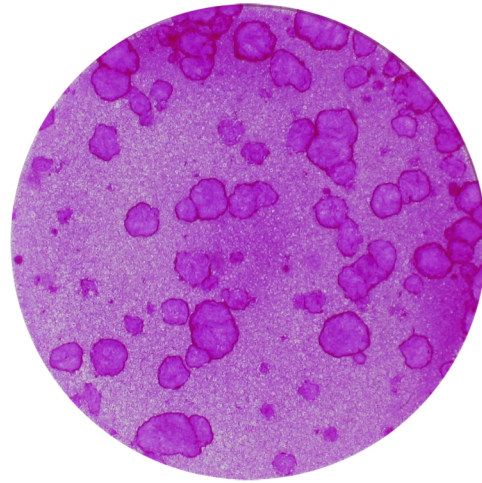
After 6 days of culture, colony forming efficiency assays were performed from conjunctival epithelial cells cultured under HCEC-HCF and HCEC-3T3 conditions. Cells cultured under HCEC-HCF conditions showed a significantly higher total colony forming efficiency compared to cells cultured under HCEC-3T3 conditions ($p = 0.002$). Also a significantly higher percentage of colonies with smooth perimeters and a surface area $> 10\text{mm}^2$ were found under HCEC-HCF- compared to HCEC-3T3 conditions ($p = 0.023$) (Fig. 26).

HCEC-HCF



Colony Forming Efficiency (CFE)
HCEC-HCF
(Representative Example)

HCEC-3T3



Colony Forming Efficiency (CFE)
HCEC-3T3
(Representative Example)

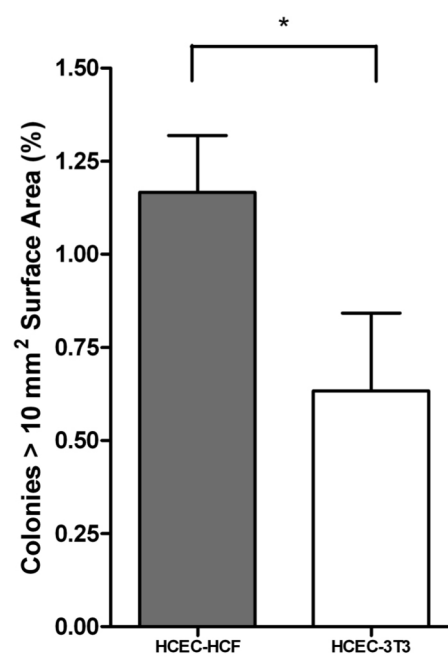
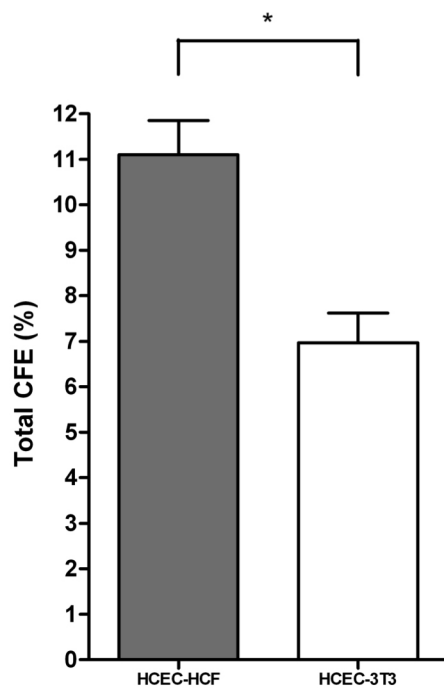


Figure 26: Comparison of the total colony forming efficiency and colonies with a surface area > 10mm² between cells cultured under HCEC-HCF and HCEC-3T3 conditions.

Cells cultured under HCEC-HCF conditions showed a significant higher total colony forming efficiency compared to cells cultured under HCEC-3T3 conditions ($p = 0.002$). Also a significantly higher percentage of colonies with smooth perimeters and a surface area > 10mm² were found

under HCEC-HCF- compared to HCEC-3T3 conditions ($p = 0.023$). All error bars represent standard deviation of the mean.

5.4. Discussion

In any conjunctival substitute a conjunctival stem cell population must be present to ensure the survival of the epithelial layer on the reconstructed ocular surface (Pellegrini, Golisano et al. 1999; Henderson and Collin 2008; Schrader, Notara et al. 2009). Stem cells obtained from a small biopsy of normal conjunctiva must be expanded *in vitro* before transplantation. Epithelial stem cells can undergo symmetric cell divisions (both daughter cells remain stem cells) to replenish the stem cell pool or can divide asymmetrically giving rise to one stem cell (to maintain the stem cell pool) and one transient amplifying cell. The latter can undergo further limited rounds of cell division to populate the cell layers of the constantly renewing conjunctival epithelium (Spradling, Drummond-Barbosa et al. 2001; Smith 2006). Stem cells are regulated by intercellular interactions, the external environment and the underlying mesenchyme forming microenvironments known as stem cell niches (Revoltella, Papini et al. 2007) and it has been shown that these tissue specific niche environments are essential for stem cell maintenance and the control of the epithelial cell-fate determination (Spradling, Drummond-Barbosa et al. 2001; Blanpain, Horsley et al. 2007).

Interactions between the mesenchyme and the overlying epithelium are an essential part of the niche environment and stromal fibroblasts seem to be strongly involved in the regulation of progenitor epithelial cell differentiation, as

they provide growth factors and cytokines that are the origin of complex paracrine and autocrine interactions between keratinocytes and fibroblasts in the niche environment (Li and Tseng 1995; Blanpain, Horsley et al. 2007).

In this study, conjunctival epithelial cells co-cultured with conjunctival fibroblasts in this serum free co-culture model, showed a high expression of the putative progenitor cell markers p63 α and ABCG2. The colony forming efficiency assays showed that cells cultured under HCEC-HCF conditions were able to produce smooth colonies containing small and tightly packed cells which had a significant higher total colony forming capacity compared to cells cultured under HCEC-3T3 conditions. Also the number of colonies with a surface area > 10mm², referred to as holoclone colonies (Barrandon and Green 1987) were significantly higher in the HCEC-HCF group compared to the HCEC-3T3 group indicating that this artificial niche environment (HCEC-HCF) is superior in maintaining a progenitor cell population compared to the standard co-culture conditions (HCEC-3T3).

We also examined CK15 as a potential marker for conjunctival progenitor cells. CK15 has been proposed as a marker for hair follicle progenitor cells (Lyle, Christofidou-Solomidou et al. 1998), but is also expressed by limbal epithelial progenitor cells (Blazejewska, Schlotzer-Schrehardt et al. 2009). In our study co-localisation of CK15 and p63 α revealed a subpopulation of CK15 positive cells inside the population of p63 α positive cells under HCEC-3T3 conditions, however under HCEC-HCF conditions only a few CK15 positive cells were found. This stands in contrast to the high expression of p63 α and ABCG2 under HCEC-HCF conditions and the higher colony forming efficiency

observed in the HCEC-HCF environment compared to the HCEC-3T3 environment. However, when the epithelial cells were further transferred after the culture period in the HCEC-HCF co-culture system onto a separate dish and then cultured under standard conditions, the emerging colonies showed strong CK15 positivity. This may show a limit for the usefulness of CK15 as a marker for conjunctival progenitor cells. Alternatively, as some cytokeratin 15 positive cells were present under HCEC-HCF conditions it may be possible that in this environment only very early progenitor cells express CK15, whereas under standard conditions (HCEC-3T3) it may also be expressed by more differentiated cells. Whilst the quantification by immunostaining and semiquantitative PCR is limited, all repetitions using these techniques showed the same trend.

The conjunctival epithelium consists of two different cell types, non-goblet and goblet cells. Goblet cells secrete mucins, which are essential for the tear film and ocular surface stability. In normal individuals the density of goblet cells is approximately 8.84 ± 4.66 goblet cells/mm as described by Ralph et al. (Ralph 1975). The goblet cell density has shown to be decreased in diseases like keratoconjunctivitis sicca, ocular pemphigoid and Stevens-Johnson syndrome (Kinoshita, Kiorpes et al. 1983). However, tear mucin content seems to show minimal variation over a greater variation in goblet cell density, indicating that a lower goblet cell density might still be sufficient to produce enough mucins for the tear film (Kinoshita, Kiorpes et al. 1983).

Previous studies have suggested the presence of a bipotent conjunctival precursor cell that is able to give rise to both non-goblet and goblet cells (Wei,

Lin et al. 1997; Pellegrini, Golisano et al. 1999) and that the commitment to differentiate into goblet cells occurs relatively late, so that goblet cells are preferentially generated by more differentiated transient amplifying cells (Pellegrini, Golisano et al. 1999). However this is not supported by all studies, as in a study by Tsai et al. the culture of conjunctival epithelial cells on 3T3 feeder cell containing collagen gels resulted in a more differentiated and stratified epithelium compared to epithelial cells cultured on collagen gels containing conjunctival fibroblasts, but goblet cell differentiation was only observed in the less stratified epithelium cultured in the presence of conjunctival fibroblasts (Tsai, Ho et al. 1994).

The presence of goblet cells has been shown *in vitro* under serum containing (Pellegrini, Golisano et al. 1999; Shatos, Rios et al. 2003) and serum free culture conditions (Ang, Tan et al. 2004). In our experiments we found single cells and groups of MUC5AC positive and PAS positive cells under HCEC-3T3 culture conditions, whereas under HCEC-HCF conditions these cells were only very rarely found. As goblet cell differentiation is supposed to occur preferentially in more differentiated transient amplifying cells, these results indicate that the HCEC-HCF condition may be superior in maintaining a more undifferentiated cell population compared to the HCEC-3T3 condition.

In conclusion, mimicking a “niche” environment *in vitro* by co-culturing mitotically active conjunctival fibroblasts with conjunctival epithelial cells supports the maintenance of conjunctival cells with progenitor cell characteristics and therefore might be a useful tool to expand conjunctival epithelial cells with progenitor cell characteristics *in vitro* for clinical use and to

investigate cell-cell interactions between fibroblasts and epithelial cells in an *in vitro* niche environment model.

Chapter 6:

**Wnt signaling in an *in vitro* niche model for
conjunctival progenitor cells**

6. Wnt signaling in an *in vitro* niche model for conjunctival progenitor cells

6.1. Introduction

It is well known that the differentiation pathways are largely determined by local micro-environment signals most of which still remain to be elucidated. In recent years, there has been considerable progress in identifying the signaling pathways that regulate the epithelial progenitor cell compartment and the Wnt signaling pathway is considered to be critical (Andl, Reddy et al. 2002; Lowry, Blanpain et al. 2005; Blanpain, Horsley et al. 2007; Watt, Estrach et al. 2008).

As the evaluation of cell-cell and cell-matrix interactions in the niche environment *in vivo* can be difficult and for a better understanding of the important niche components, the development of *in-vitro* models may be useful. As described in the previous chapter our group has recently shown that mimicking a “niche” environment *in vitro*, by co-culturing ocular surface epithelial cells with mitotically active fibroblasts from the respective tissue in a serum free culture system, supports the maintenance of epithelial progenitor cells (Notara, Shortt et al. 2010; Schrader, Notara et al. 2010).

The aim of this study was to further investigate this system and to identify stem cell maintenance and differentiation related pathways. To identify and explore biological pathway profiles of conjunctival epithelial cells in this *in vitro* niche environment, microarray gene analysis and quantitative RT-PCR was performed and the gene expression profiles were compared to the standard culture technique which uses 3T3 mouse feeder cells and foetal calf serum.

6.2. Methods

The methods used for the experiments in this chapter are described in chapter 2 (Materials and Methods). Conjunctival cells from six conjunctival biopsies from six different donors were used for the experiments and each experiment was repeated three times. The methods specific to this chapter are described below.

6.2.1. Analysis of gene expression by Affymetrix GeneChip® human genome arrays

6.2.1.1. The principles of microarray technology

The human genomic library is composed of 20,000 - 25,000 genes (as of September 2010 the NCBI record contains 23,739 fully identified coding sequences), which are coded by particular segments along specific DNA strands (Akasaka, Ono et al. 2010). This library provides the blueprint for the creation of functional proteins, with copies of these blueprints (genes) made by intracellular machinery to form messenger RNA (mRNA). The code contained within the sequence of mRNA is translated by the ribosome to create functional proteins. The regulation of the translational process from gene to protein is otherwise known as gene expression; an upregulation in gene expression typically, however not always, results in an increase in protein, whereas a downregulation in gene expression typically results in a decrease in protein (Bichindaritz 2010). Various tools are available to scientists to understand the functional state of a cell, whether it is in a diseased state or

following drug treatment, through interrogation at either the genomic or protein level; however, many of these require the investigation of only one gene or protein per experiment. In 1995, Schena and colleagues published a paper that changed scientists approach to understanding the changes in gene expression: the cDNA microarray (Schena, Shalon et al. 1995). Whilst microarrays had been under development for sometime, the publication of an in-house produced microarray allowed scientists to rapidly investigate the expression profiles of multiple genes in single experiments, at relatively low cost following initial set up (Southern 2001).

6.2.1.2. Affymetrix oligonucleotide arrays

The affymetrix oligonucleotide array is generated by the in situ synthesis of oligonucleotides through photolithography (Fodor, Rava et al. 1993; Pease, Solas et al. 1994). This “on chip” printing of small sized probes has allowed for the generation of arrays with multiple probes per single gene. The current GeneChip Human Gene 1.0 ST Array, developed by Affymetrix and designed for human gene expression analysis, contains probe sets (a group of probes that are designed for a single gene) covering 28,869 identified human genes [though some of these are now redundant as the genome has been updated – the latest Affymetrix GeneChips are based upon the publications of Human Genome revision 36 (Karolchik, Kuhn et al. 2008)]. Each GeneChip contains a median number of 26 distinct probes per gene (that form the probe set), resulting in 764,885 distinct probes across the array with each probe 25 base

pairs in size. The GeneChip platform analyses only one sample per array and the arrays measure absolute mRNA levels of single samples, using a single fluorescent dye, and require multiple chips to compare between samples.

6.2.1.3. Sample processing and steps of the expression array

6.2.1.3.1 RNA isolation and quality control

To ensure pure epithelial cell samples were obtained from both groups for the analysis, a two step trypsinisation method was used as described in chapter 5.2.3. Total RNA was isolated from HCEC cultured under HCEC-HCF and HCEC-3T3 conditions using the RNeasy system (Qiagen, Germany) according to the manufacturer's instructions. Residual genomic DNA was eliminated from the samples by On-Column DNase digestion (RNase-Free DNase Set, Qiagen, Germany) and the samples were stored at -80°C . The quality of these RNA samples was assessed using a Nanodrop ND-1000 (Thermo Scientific, UK) which provided a concentration of RNA ($\text{ng}/\mu\text{l}$) as well as a determination of the purity of the RNA through a measure of the absorbance of the solution at 260 nm (nucleic acids) and 280 nm (proteins and phenol) and a ratio between the two calculated; only samples with a 260/280 ratio of 1.8-2.1 were used for further experimentation.

Additionally, prior to the microarray experiments, the quality of the RNA was assessed by the Agilent 2100 Bioanalyzer at the Bloomsbury Centre for Bioinformatics at University College London (UCL), to check for the confirmation of the two ribosomal peaks (18s and 28s) together with good

separation between each peak. From the results the software calculated the RNA concentration as well as an RNA Integrity Number (RIN), determining the quality of RNA.

6.2.1.3.2 Hybridization and array scanning

Affymetrix GeneChip® Human Gene 1.0 ST Array was performed by the Bloomsbury Centre for Bioinformatics at University College London (UCL) according to the manufacturer's protocols (Affymetrix 2009). In brief, total RNA was reverse transcribed to cDNA, *in vitro* transcribed to cRNA, fragmented and Biotin-labeled. Then hybridization of the fragmented Biotin-labeled cRNA with the Affymetrix GeneChip® expression array was performed. In order to generate signals the Affymetrix platform uses phycoerythrin labelled streptavidin (SAPE) to bind to the previously biotinylated cRNA using the Fluidics Station 450. After washing and staining steps, the chip was scanned and quantified (Fig. 27).

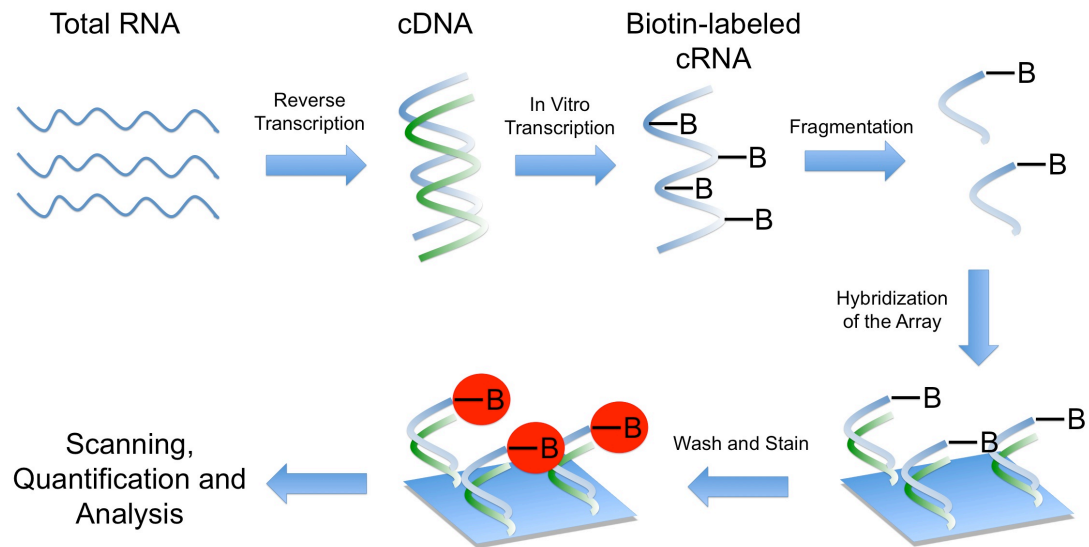


Figure 27: Steps of the Affymetrix GeneChip whole transcript sense target labeling assay (image adapted from affymetrix.com)

6.2.1.3.3 Microarray data analysis

The intensity information from each probe within a microarray must first be combined together to produce expression values, which can then be used to determine gene expression. For the QC of Affymetrix data the RMA algorithm was used to perform the background adjustment, normalization and summarization.

After that, the microarray analysis was done using LIMMA, a Bioconductor package for differential expression analysis (<http://www.bioconductor.org>). In a 2 group comparison, LIMMA applies a modified t-test which uses a Bayesian approach to make the analysis stable even for small sample numbers. The Benjamini-Hochberg-corrected p-value cut-off of 0.05 was used to select significantly differential gene expression.

6.2.1.3.4 Gene ontology (GO) and pathway profile analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7) was used to identify significant biological clusters, processes and pathways represented in the sample of genes that met the requirement for differential expression ($p < 0.05$) (Dennis, Sherman et al. 2003). In the calculation of gene groups, a minimum of 2 genes were required with an EASE score (a modified Fisher Exact p-value) of less than 0.05. The biological clustering was based upon the gene ontology (GO) terms available in the GO library. The significantly altered biological clusters were sorted in descending order by the number of genes involved in the biological processes (BP's) and out of the top 25% BP's the 10 most relevant to cell proliferation, cell survival and cell death were chosen. Pathvisio (<http://www.pathvisio.org>) (van Iersel, Kelder et al. 2008) and wikipathways (<http://www.wikipathways.org>) (Pico, Kelder et al. 2008) were used to view whole human genome data on biological pathways in order to identify changes between HCEC-HCF and HCEC-3T3 conditions associated with stem cell maintenance and differentiation.

6.2.2. Reverse transcription (RT) and quantitative real-time PCR

The quality of these RNA samples was assessed using a Nanodrop ND-1000. For the RT-reaction, 1 μg of total RNA was reverse-transcribed in 20- μl reactions consisting of 5 mM MgCl_2 , 1 mM deoxynucleoside-5'-triphosphate (dNTP), 1 U/ μl RNase inhibitor, 0.8 U/ μl AMV reverse transcriptase (Roche

Diagnostics, Switzerland), and 80 ng/μl oligo(dT)-15 primers (Roche) in 10 mM Tris/HCl buffer (pH=7.4) containing 50 mM KCl. The mixture was incubated as follows: 10 minutes at 25°C, 60 minutes at 42°C, 5 minutes at 99°C, and 5 minutes at 4°C in a thermal cycler (Eppendorf AG). Quantitative PCR reactions were performed with a SYBR green nucleic acid staining kit (SABiosciences) in 96-well plates (SABiosciences human Wnt signaling PCR-array interrogating 84 Wnt pathway related genes (PAHS-043)) in a sequence-detection system (Applied Biosystems [ABI] 7900HT). The interrogated genes are shown in table 3. The results were analysed by the comparative threshold cycle (CT) method and normalised by 4 housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 60S ribosomal protein L13a (RPL13A), Hypoxanthine-guanine phosphoribosyltransferase (HPRT1) and Beta-actin (ACTB)). The Student's unpaired t-test was used to compare the two conditions and sets of data producing $p \leq 0.05$ were considered statistically significant. All the experiments were performed in triplicate.

| UniGene | RefSeq | Symbol | Description |
|-----------|-----------|---------|--|
| Hs.515053 | NM_001130 | AES | Amino-terminal enhancer of split |
| Hs.158932 | NM_000038 | APC | Adenomatous polyposis coli |
| Hs.592082 | NM_003502 | AXIN1 | Axin 1 |
| Hs.415209 | NM_004326 | BCL9 | B-cell CLL/lymphoma 9 |
| Hs.643802 | NM_033637 | BTRC | Beta-transducin repeat containing |
| Hs.17631 | NM_003468 | FZD5 | Frizzled homolog 5 (Drosophila) |
| Hs.523852 | NM_053056 | CCND1 | Cyclin D1 |
| Hs.376071 | NM_001759 | CCND2 | Cyclin D2 |
| Hs.534307 | NM_001760 | CCND3 | Cyclin D3 |
| Hs.529862 | NM_001892 | CSNK1A1 | Casein kinase 1, alpha 1 |
| Hs.631725 | NM_001893 | CSNK1D | Casein kinase 1, delta |
| Hs.646508 | NM_022048 | CSNK1G1 | Casein kinase 1, gamma 1 |
| Hs.644056 | NM_001895 | CSNK2A1 | Casein kinase 2, alpha 1 polypeptide |
| Hs.208597 | NM_001328 | CTBP1 | C-terminal binding protein 1 |
| Hs.501345 | NM_022802 | CTBP2 | C-terminal binding protein 2 |
| Hs.476018 | NM_001904 | CTNNB1 | Catenin (cadherin-associated protein), beta 1, 88kDa |
| Hs.463759 | NM_020248 | CTNBP1 | Catenin, beta interacting protein 1 |
| Hs.12248 | NM_025212 | CXXC4 | CXXC finger 4 |

| | | | |
|-----------|--------------|----------|---|
| Hs.654934 | NM_014992 | DAAM1 | Dishevelled associated activator of morphogenesis 1 |
| Hs.655626 | NM_033425 | DIXDC1 | DIX domain containing 1 |
| Hs.40499 | NM_012242 | DKK1 | Dickkopf homolog 1 (Xenopus laevis) |
| Hs.74375 | NM_004421 | DVL1 | Dishevelled, dsh homolog 1 (Drosophila) |
| Hs.118640 | NM_004422 | DVL2 | Dishevelled, dsh homolog 2 (Drosophila) |
| Hs.517517 | NM_001429 | EP300 | E1A binding protein p300 |
| Hs.484138 | NM_012300 | FBXW11 | F-box and WD repeat domain containing 11 |
| Hs.494985 | NM_012164 | FBXW2 | F-box and WD repeat domain containing 2 |
| Hs.1755 | NM_002007 | FGF4 | Fibroblast growth factor 4 |
| Hs.283565 | NM_005438 | FOSL1 | FOS-like antigen 1 |
| Hs.663679 | NM_003593 | FOXN1 | Forkhead box N1 |
| Hs.126057 | NM_005479 | FRAT1 | Frequently rearranged in advanced T-cell lymphomas |
| Hs.128453 | NM_001463 | FRZB | Frizzled-related protein |
| Hs.36975 | NM_000510 | FSHB | Follicle stimulating hormone, beta polypeptide |
| Hs.94234 | NM_003505 | FZD1 | Frizzled homolog 1 (Drosophila) |
| Hs.142912 | NM_001466 | FZD2 | Frizzled homolog 2 (Drosophila) |
| Hs.40735 | NM_017412 | FZD3 | Frizzled homolog 3 (Drosophila) |
| Hs.19545 | NM_012193 | FZD4 | Frizzled homolog 4 (Drosophila) |
| Hs.591863 | NM_003506 | FZD6 | Frizzled homolog 6 (Drosophila) |
| Hs.173859 | NM_003507 | FZD7 | Frizzled homolog 7 (Drosophila) |
| Hs.302634 | NM_031866 | FZD8 | Frizzled homolog 8 (Drosophila) |
| Hs.466828 | NM_019884 | GSK3A | Glycogen synthase kinase 3 alpha |
| Hs.445733 | NM_002093 | GSK3B | Glycogen synthase kinase 3 beta |
| Hs.714791 | NM_002228 | JUN | Jun oncogene |
| Hs.229335 | NM_001039570 | KREMEN1 | Kringle containing transmembrane protein 1 |
| Hs.555947 | NM_016269 | LEF1 | Lymphoid enhancer-binding factor 1 |
| Hs.6347 | NM_002335 | LRP5 | Low density lipoprotein receptor-related protein 5 |
| Hs.584775 | NM_002336 | LRP6 | Low density lipoprotein receptor-related protein 6 |
| Hs.202453 | NM_002467 | MYC | V-myc myelocytomatosis viral oncogene homolog (avian) |
| Hs.592059 | NM_033119 | NKD1 | Naked cuticle homolog 1 (Drosophila) |
| Hs.208759 | NM_016231 | NLK | Nemo-like kinase |
| Hs.643588 | NM_000325 | PITX2 | Paired-like homeodomain 2 |
| Hs.386453 | NM_022825 | PORCN | Porcupine homolog (Drosophila) |
| Hs.483408 | NM_002715 | PPP2CA | Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform |
| Hs.467192 | NM_014225 | PPP2R1A | Protein phosphatase 2 (formerly 2A), regulatory subunit A, alpha isoform |
| Hs.256587 | NM_015617 | PYGO1 | Pygopus homolog 1 (Drosophila) |
| Hs.647774 | NM_021205 | RHOU | Ras homolog gene family, member U |
| Hs.401388 | NM_021627 | SEN2P | SUMO1/sentrin/SMT3 specific peptidase 2 |
| Hs.713546 | NM_003012 | SFRP1 | Secreted frizzled-related protein 1 |
| Hs.658169 | NM_003014 | SFRP4 | Secreted frizzled-related protein 4 |
| Hs.500822 | NM_022039 | FBXW4 | F-box and WD repeat domain containing 4 |
| Hs.396783 | NM_004252 | SLC9A3R1 | Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1 |
| Hs.98367 | NM_022454 | SOX17 | SRY (sex determining region Y)-box 17 |
| Hs.389457 | NM_003181 | T | T, brachyury homolog (mouse) |
| Hs.573153 | NM_003202 | TCF7 | Transcription factor 7 (T-cell specific, HMG-box) |
| Hs.516297 | NM_031283 | TCF7L1 | Transcription factor 7-like 1 (T-cell specific, HMG-box) |
| Hs.197320 | NM_005077 | TLE1 | Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila) |
| Hs.332173 | NM_003260 | TLE2 | Transducin-like enhancer of split 2 (E(sp1) homolog, Drosophila) |
| Hs.284122 | NM_007191 | WIF1 | WNT inhibitory factor 1 |
| Hs.492974 | NM_003882 | WISP1 | WNT1 inducible signaling pathway protein 1 |
| Hs.248164 | NM_005430 | WNT1 | Wingless-type MMTV integration site family, member 1 |
| Hs.121540 | NM_025216 | WNT10A | Wingless-type MMTV integration site family, member 10A |
| Hs.108219 | NM_004626 | WNT11 | Wingless-type MMTV integration site family, member 11 |
| Hs.272375 | NM_057168 | WNT16 | Wingless-type MMTV integration site family, member 16 |
| Hs.567356 | NM_003391 | WNT2 | Wingless-type MMTV integration site family member 2 |

| | | | |
|-----------|-----------|-------|---|
| Hs.258575 | NM_004185 | WNT2B | Wingless-type MMTV integration site family, member 2B |
| Hs.445884 | NM_030753 | WNT3 | Wingless-type MMTV integration site family, member 3 |
| Hs.336930 | NM_033131 | WNT3A | Wingless-type MMTV integration site family, member 3A |
| Hs.25766 | NM_030761 | WNT4 | Wingless-type MMTV integration site family, member 4 |
| Hs.696364 | NM_003392 | WNT5A | Wingless-type MMTV integration site family, member 5A |
| Hs.306051 | NM_032642 | WNT5B | Wingless-type MMTV integration site family, member 5B |
| Hs.29764 | NM_006522 | WNT6 | Wingless-type MMTV integration site family, member 6 |
| Hs.72290 | NM_004625 | WNT7A | Wingless-type MMTV integration site family, member 7A |
| Hs.512714 | NM_058238 | WNT7B | Wingless-type MMTV integration site family, member 7B |
| Hs.591274 | NM_058244 | WNT8A | Wingless-type MMTV integration site family, member 8A |
| Hs.149504 | NM_003395 | WNT9A | Wingless-type MMTV integration site family, member 9A |

Table 3: Genes table of interrogated genes by the SABiosciences human Wnt signaling PCR-array (PAHS-043)

6.3. Results

6.3.1. Affymetrix microarray analysis

6.3.1.1. RNA quality control results

Before the microarray experiments, each RNA sample was individually checked for the confirmation of the two ribosomal peaks (18s and 28s) together with good separation between each peak, using the Agilent Bioanalyzer. From the results the software was also able to calculate the RNA concentration as well as an RNA Integrity Number (RIN), determining the quality of RNA on a 1-10 scale for any one individual sample, with scores ≥ 8.0 being adequate for microarray analysis. The RIN score is calculated from the entire electrophoretic trace rather than just the ratio of ribosomal bands and therefore allows the presence or absence of any degradation products to be detected. All six samples passed the RNA quality control (Fig. 28).

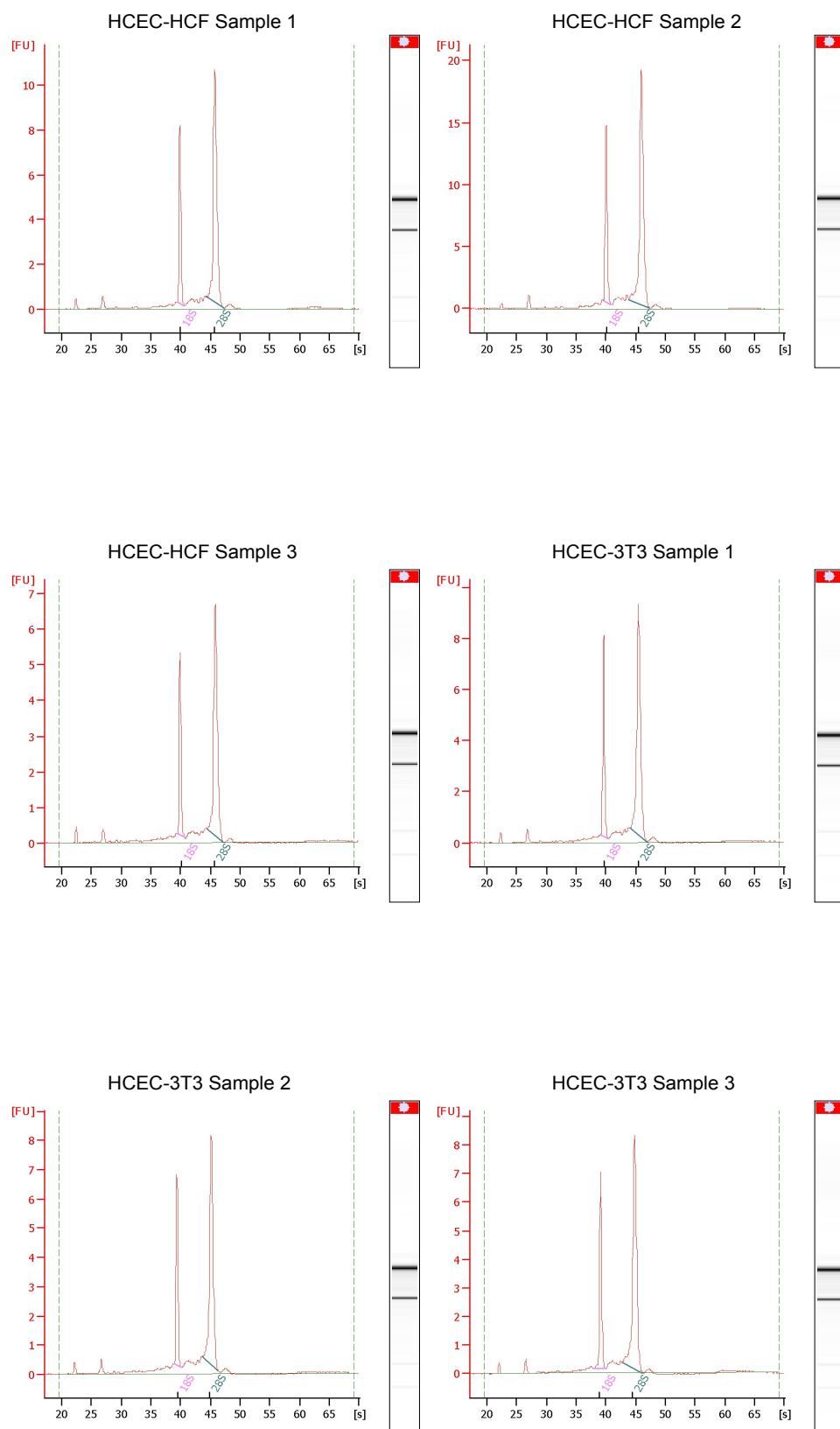


Figure 28: RNA quality control by the Agilent Bioanalyzer for the samples

from the HCEC-HCF and HCEC-3T3 (control) group.

The electrophoretic traces show the two ribosomal peaks (18s and 28s) together with good separation between each peak, indicating the absence of degradation products.

6.3.1.2. Microarray quality control results

Following hybridisation and scanning, a series of quality control checks were performed on the microarrays. The individual samples were labelled as shown in table 4.

| Chip | Name | Condition |
|-------------------|-------------------|-----------------------------|
| G302_HCC_HF1.CEL | HCEC-HCF Sample 1 | <i>In Vitro</i> Niche Model |
| G302_HCC_HF2.CEL | HCEC-HCF Sample 2 | <i>In Vitro</i> Niche Model |
| G302_HCC_HF3.CEL | HCEC-HCF Sample 3 | <i>In Vitro</i> Niche Model |
| G302_HCC_3T31.CEL | HCEC-3T3 Sample 1 | Control |
| G302_HCC_3T32.CEL | HCEC-3T3 Sample 2 | Control |
| G302_HCC_3T33.CEL | HCEC-3T3 Sample 3 | Control |

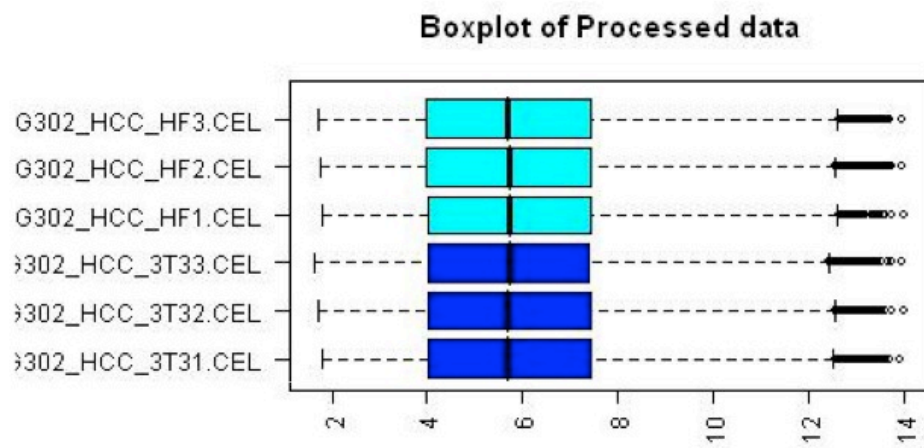
Table 4: File- and sample names for the HCEC-HCF and the HCEC-3T3 (control) group.

Pre-processing of Affymetrix data involved three steps: background adjustment, normalisation and summarisation. Normalisation adjusts the data to make the measurements from different arrays comparable. Summarisation combines the multiple probe intensities for each probeset to produce a single expression value for each probe set. The Robust Multi-Array Analysis (RMA)

algorithm was used to get the normalised data and gene signals. This method performs within-chip and between-chip normalisations in a single step.

Box-plots were generated showing the range of intensity values, ideally all the boxes should look the same; having the same median and showing similar intensity range. The QC results showed, that the samples from both groups showed similar inter-quartile ranges and medians (Fig. 29A). The intensity distribution graph shows the density of the probe intensities and the distributions of the intensities overlapped well between the arrays (Fig. 29B). The MA plots assess the reproducibility of biological replicates providing an indication of whether to expect large differences in gene expression between the samples within each group. The graph is composed of the y-axis, representing the M-value (\log_2 fold change in the intensity of each probe set from each chip of the two samples), with the x-axis representing the A values, or the average intensity of each probe set across the chips as obtained during normalisation. The plots showed symmetry around the x-axis. Furthermore, at high intensities the points are close to the x-axis, indicating good agreement between the replicates (Yang, Dudoit et al. 2002) (Fig. 30A+B). As most samples within the same group are expected to have similar gene expression profiles most genes should lie around 0 (no change in gene expression). As the signal intensity decreases, increased values for M are to be expected as variation in intensity has a much greater effect on the M value. Outliers are further expected due to the number of data points that are being investigated.

A



B

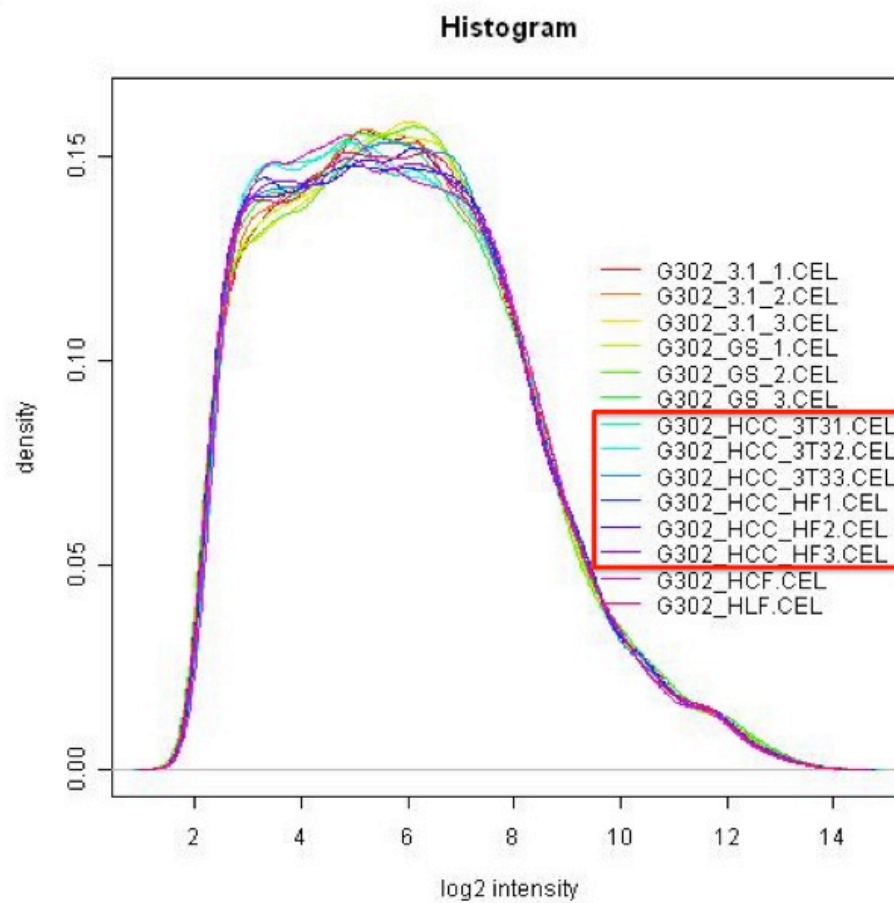
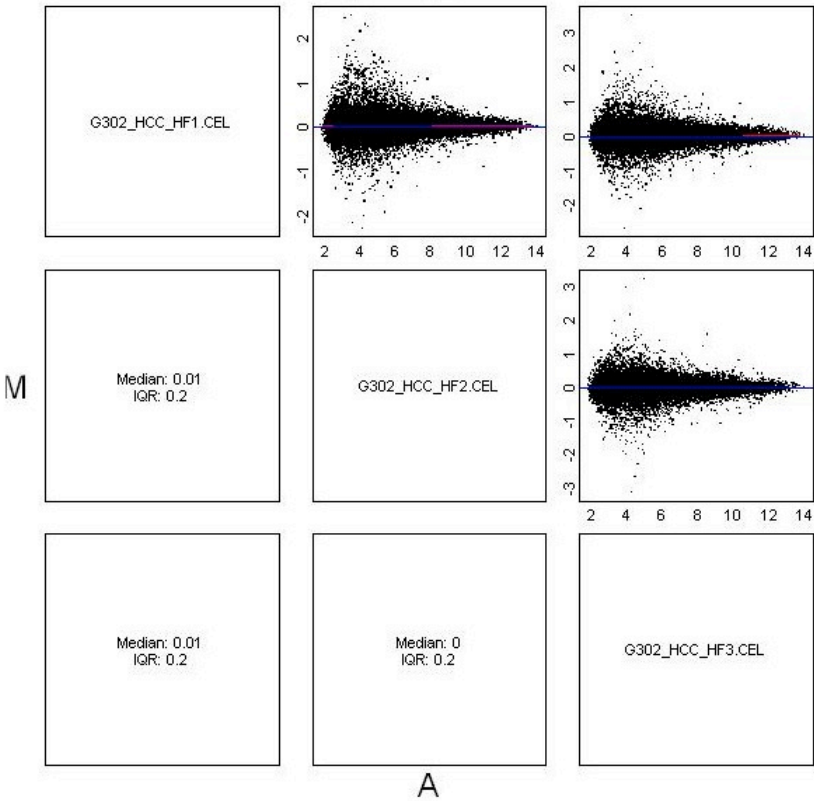


Figure 29: Quality control of the microarray experiments.

A: Box plots, showing the range of intensity values, with similar medians and interquartile ranges in the different samples. B (red box): Probe intensities, showing that the distributions of the intensities overlap well between arrays.

A

MvA group HCEC-HCF



B

MvA group HCEC-3T3

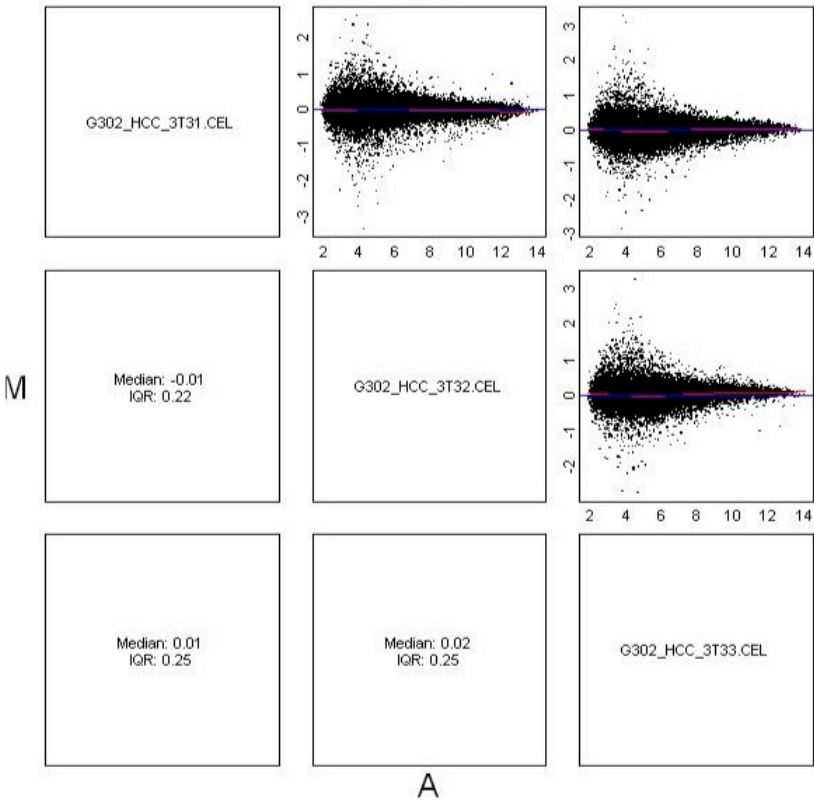
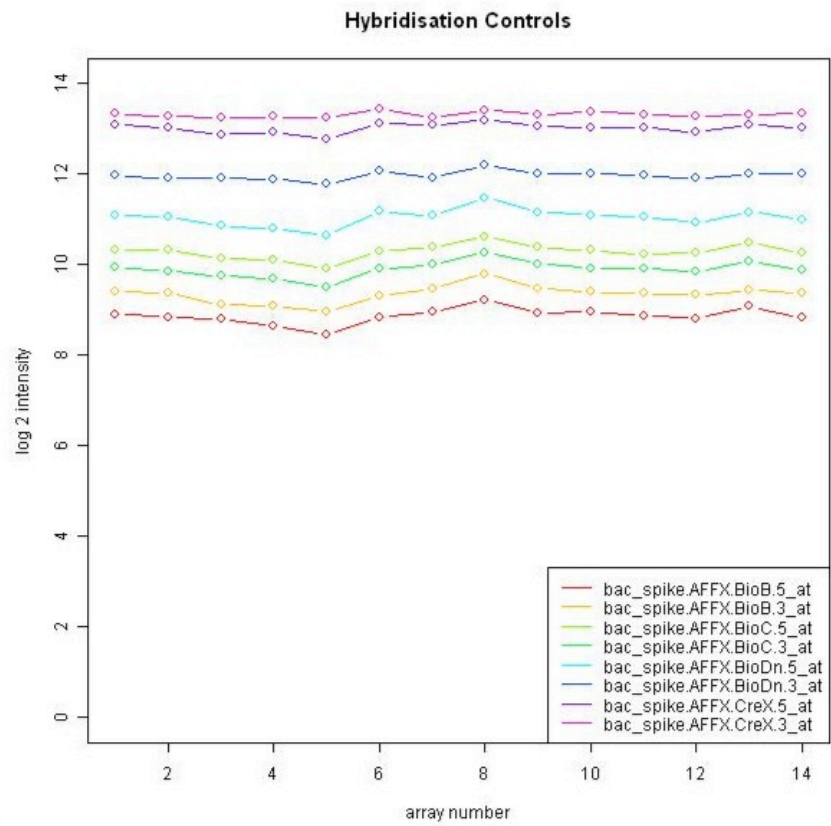


Figure 30: Quality control of the microarray experiments.

A+B: MvA plots for the HCEC-HCF and the HCEC-3T3 group . Each set of replicates are symmetric about the x-axis and indicates good agreement between biological replicates.

The control probeset analysis indicated that all samples hybridised correctly to the chip with the built in biological controls producing no errors. Bac and PolyA control probesets had similar values across samples and display the expected rank order (Fig 31A+B). All arrays had similar positive and negative control signal values and the area under the curve fell between the 0.8 and 1 range indicating a good separation between positive and negative controls (Fig. 32).

A



B

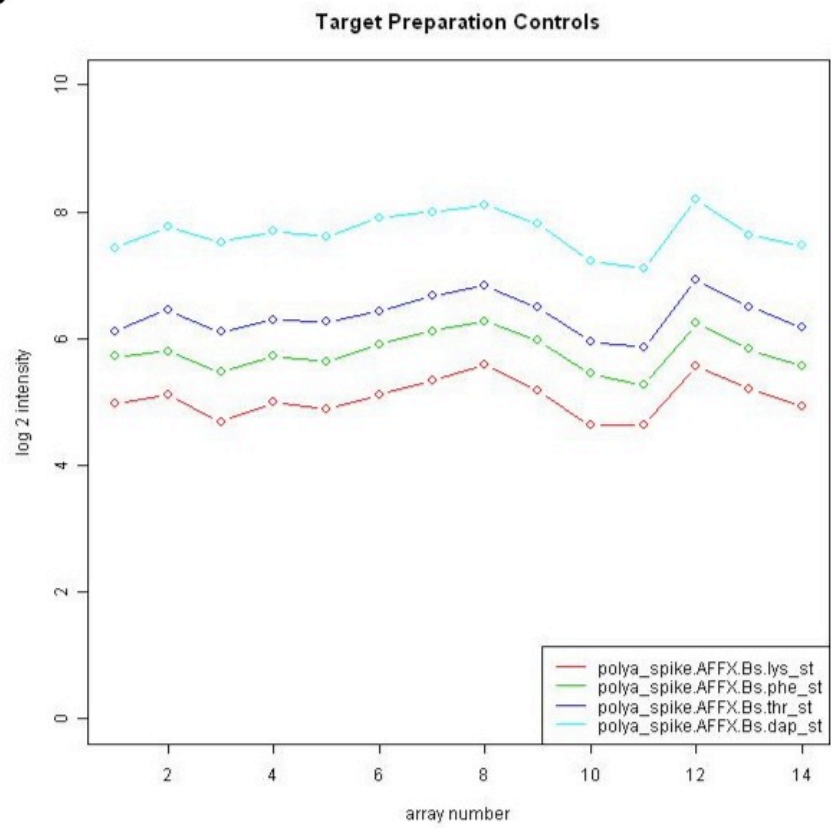


Figure 31: Quality control of the microarray experiments.

A+B: BAC and polyA probeset graphs respectively. The probesets had similar values across the samples and they display the expected rank order.

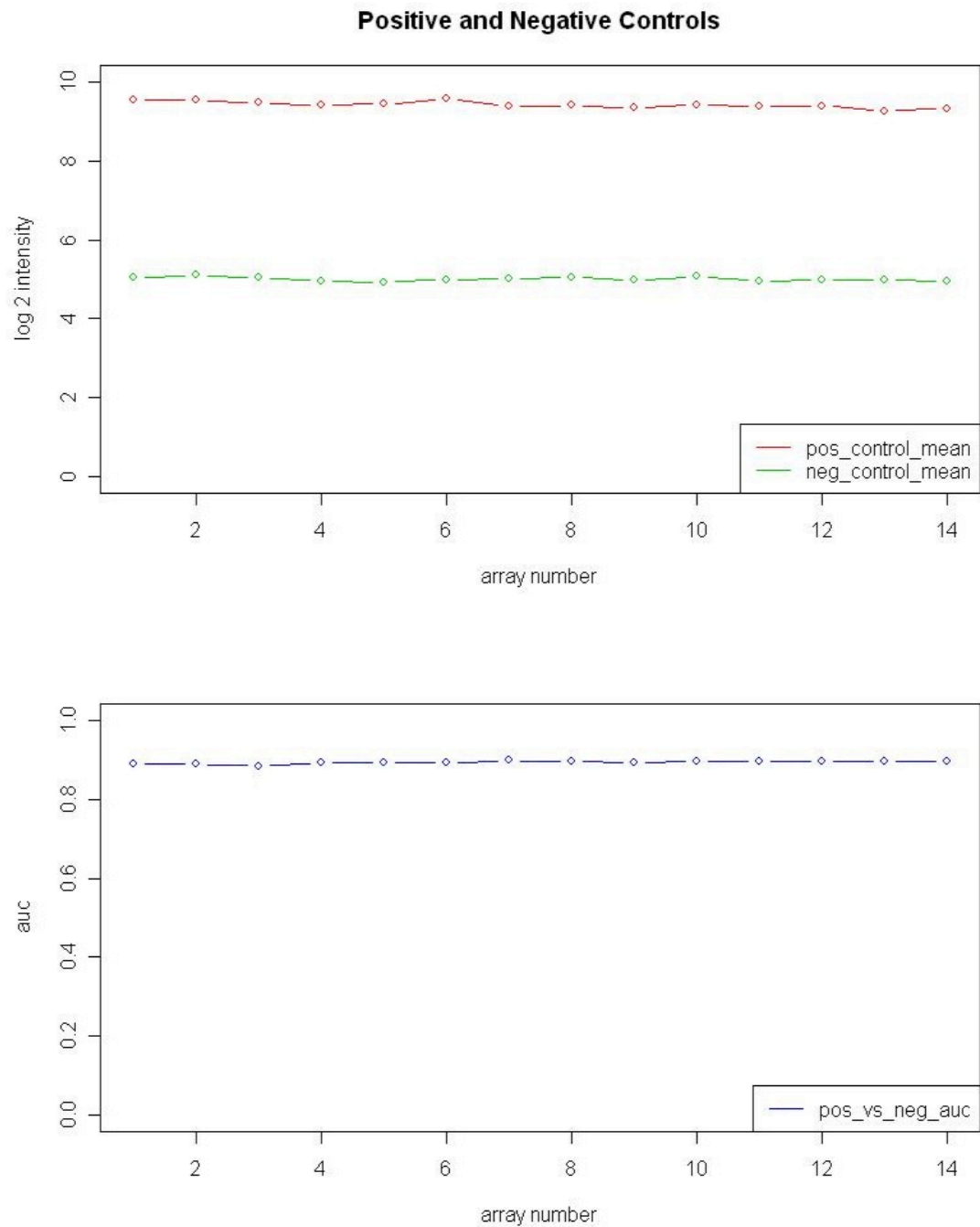


Figure 32: Plot of the positive and negative control together with an area under the curve (AUC) plot.

The AUC fell between 0.8 and 1 indicating there was good separation between the positive and negative controls.

6.3.1.3. Microarray gene expression analysis of HCEC cultured under HCEC-HCF and HCEC-3T3 conditions

The GeneChip® Human Gene 1.0 ST Array interrogated 28,869 well-annotated genes with 764,885 distinct probes. The primary analysis revealed that 2706 genes were significantly altered between HCEC-HCF and HCEC-3T3 conditions, whereby 1372 genes were upregulated and 1334 genes were downregulated in HCEC-HCF compared to HCEC-3T3 conditions (Fig. 33A).

The following clustering into biological processes using DAVID showed significant alterations ($p \leq 0.05$) of biological processes involved in the intracellular signalling cascade, regulation of cell proliferation, regulation of cell death, cell adhesion, cell death, cytoskeleton organization, cell migration, regulation of cell cycle, epithelium development and epithelial cell differentiation between HCEC-HCF and HCEC-3T3 conditions (Fig. 33B). Considering that these processes may involve a number of genes, we used less restrictive gene selection criteria that admitted any individual gene as long as its change in expression was significant ($p \leq 0.05$).

Evaluation of differences in stem cell signalling related pathways using pathvisio and wikipathways revealed that 21.7% of all genes involved in the Wnt signaling pathway, 20.6% of the genes involved in the MAPK signaling pathway, 18.6% of the genes involved in Toll-like receptor signaling pathway, 18.2% of the genes involved in Hedgehog signaling pathway, 16.3% of the

genes involved in Wnt signaling and pluripotency and 10.9% of the genes involved in Notch Signaling Pathway were altered between HCEC-HCF and HCEC-3T3 conditions (Fig 33C). The Wnt signaling pathway was chosen for further analysis because it showed the highest percentage of changes in gene expression between the two culture conditions. However, other stem cell related pathways like the MAPK signaling pathway, the Toll-like receptor signaling pathway, the Hedgehog signaling pathway and the Notch Signaling pathway also showed changes in gene expression and it would be important to look at these pathways as well in following studies.

| | | |
|----------|--|-------------|
| A | Total number of significantly altered genes (p<0.05) | 2706 |
| | Upregulated HCEC-HCF (compared to HCEC-3T3) | 1372 |
| | Downregulated HCEC-HCF (compared to HCEC-3T3) | 1334 |

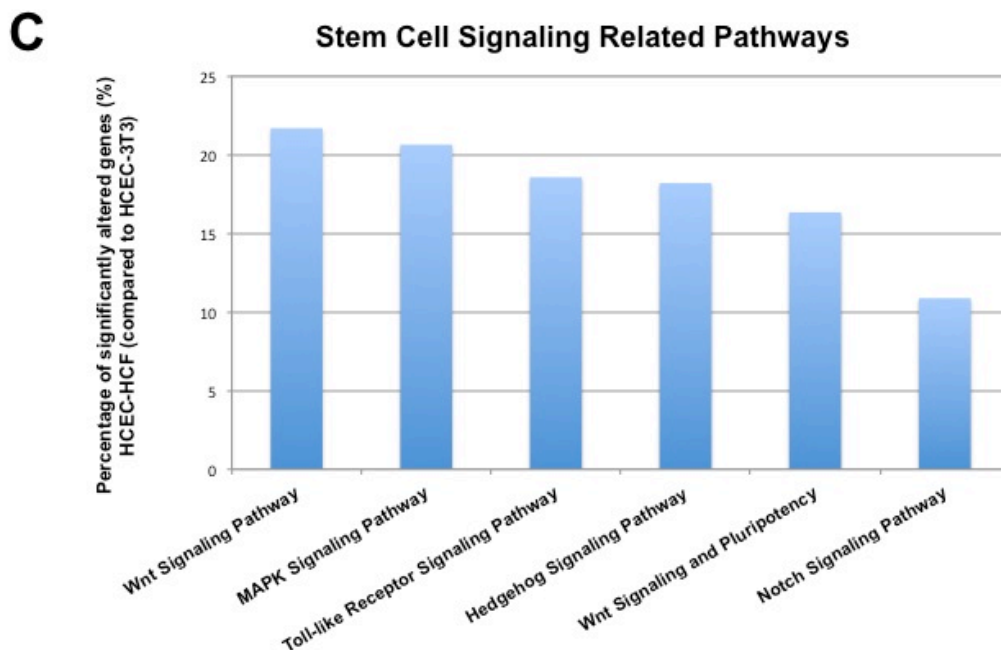
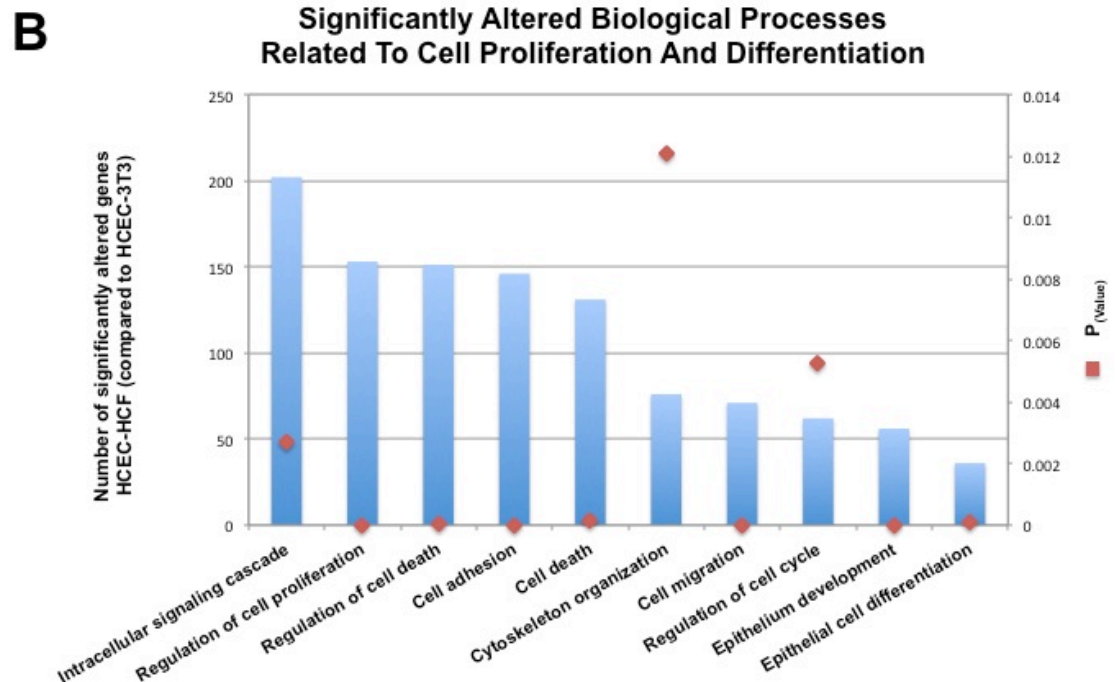


Figure 33: Microarray gene expression analysis of HCEC cultured under HCEC-HCF and HCEC-3T3 conditions.

A: Primary LIMMA analysis showing the number of significantly altered genes between culture conditions. B: Cluster analysis (DAVID), showing significant alterations ($p \leq 0.05$) of biological processes related to cell proliferation and differentiation and cell death between HCEC-HCF and HCEC-3T3 conditions. C: Evaluation of differences in stem cell signaling related pathways (using pathvisio and wikipathways) between HCEC-HCF and HCEC-3T3 conditions.

6.3.2. Analysis of differences in Wnt signaling associated gene expression between HCEC-HCF and HCEC-3T3 conditions by quantitative PCR

Interrogation of 84 Wnt signaling related genes by quantitative PCR revealed a significant difference in gene expression in 22 out of 84 tested genes (Table 5). A significant difference in gene expression and fold change (FC) ≥ 1.5 was found in 15 genes, whereby 12 genes were downregulated and 3 were upregulated under HCEC-HCF compared to HCEC-3T3 conditions. The downregulated genes included the Wnt ligands Wnt3, Wnt4, Wnt7B, Wnt10A, Wnt receptor proteins FZD1, LRP5, LRP6, β -Catenin (CTNNB1) and the Wnt target gene Cyclin D1 (CCND1) (Fig. 35).

| Gene | P-Value | FC |
|---------|---------|--------|
| AES | 0.028 | -1.31 |
| BCL9 | 0.020 | -1.62 |
| CCND1 | 0.003 | -4.65 |
| CSNK2A1 | 0.015 | 1.50 |
| CTNNB1 | 0.034 | -1.51 |
| DVL1 | 0.020 | 1.48 |
| FOXN1 | 0.012 | 2.87 |
| FRAT1 | 0.026 | -1.45 |
| FZD1 | 0.029 | -2.13 |
| GSK3B | 0.017 | 1.40 |
| JUN | 0.010 | -2.71 |
| LRP5 | 0.018 | -2.33 |
| LRP6 | 0.005 | -1.62 |
| PPP2R1A | 0.017 | -1.28 |
| SENP2 | 0.015 | -1.16 |
| TCF7L1 | 0.040 | -1.26 |
| TLE2 | 0.006 | -4.08 |
| WISP1 | 0.006 | 1.67 |
| WNT10A | 0.001 | -18.26 |
| WNT3 | 0.031 | -1.67 |
| WNT4 | 0.006 | -2.66 |
| WNT7B | 0.012 | -2.76 |

Table 5: Analysis of differences in Wnt signaling associated gene expression between HCEC-HCF and HCEC-3T3 conditions by quantitative PCR.

Interrogation of 84 Wnt signaling related genes by quantitative PCR revealed a significant difference in gene expression in 22 out of 84 tested genes ($p \leq 0.05$), whereby 17 genes were downregulated (red) and 5 genes were upregulated (green) under HCEC-HCF compared to HCEC-3T3 conditions.

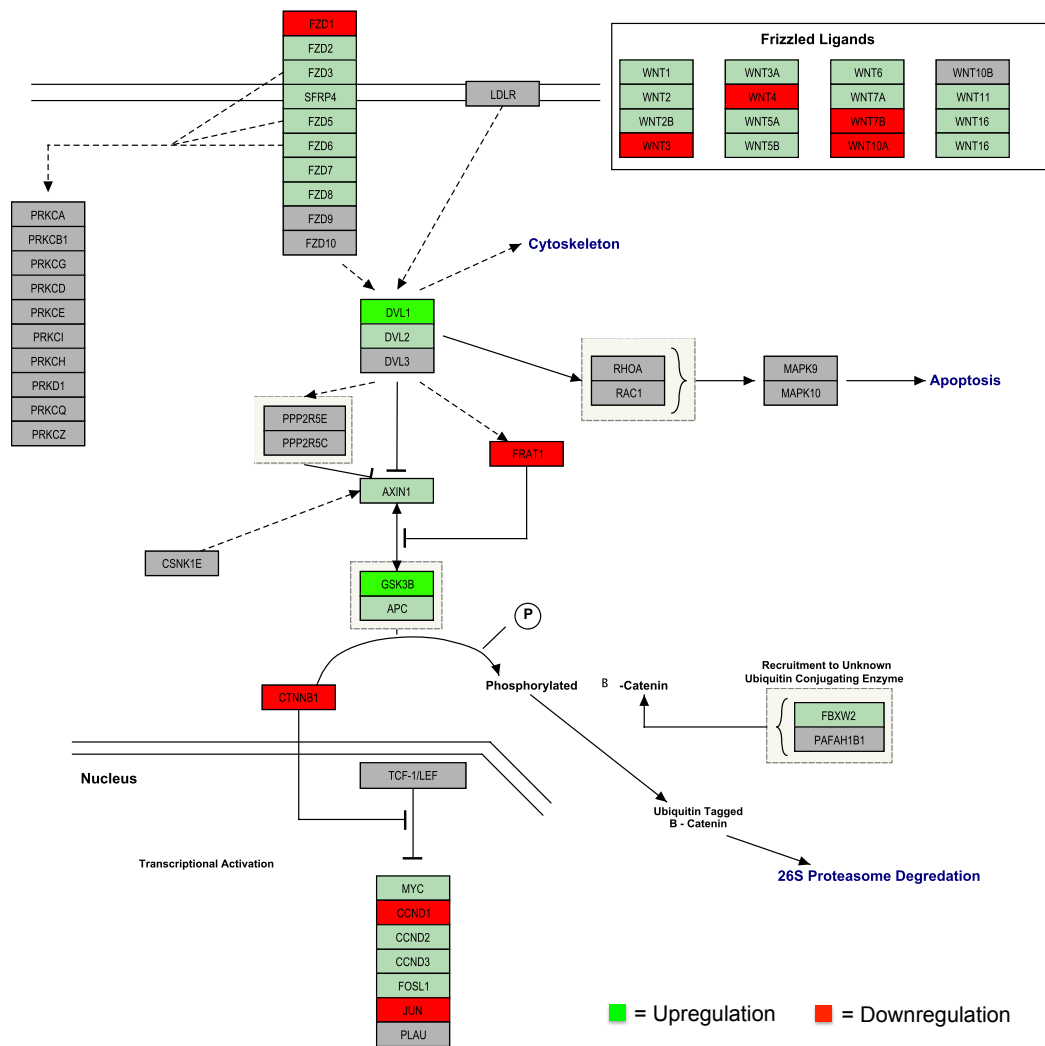


Figure 34: Scheme of the Wnt signaling pathway.

The pathway scheme shows significantly upregulated and downregulated genes ($p \leq 0.05$) in the wnt signaling pathway from conjunctival epithelial cells cultured under HCEC-HCF compared to HCEC-3T3 conditions.

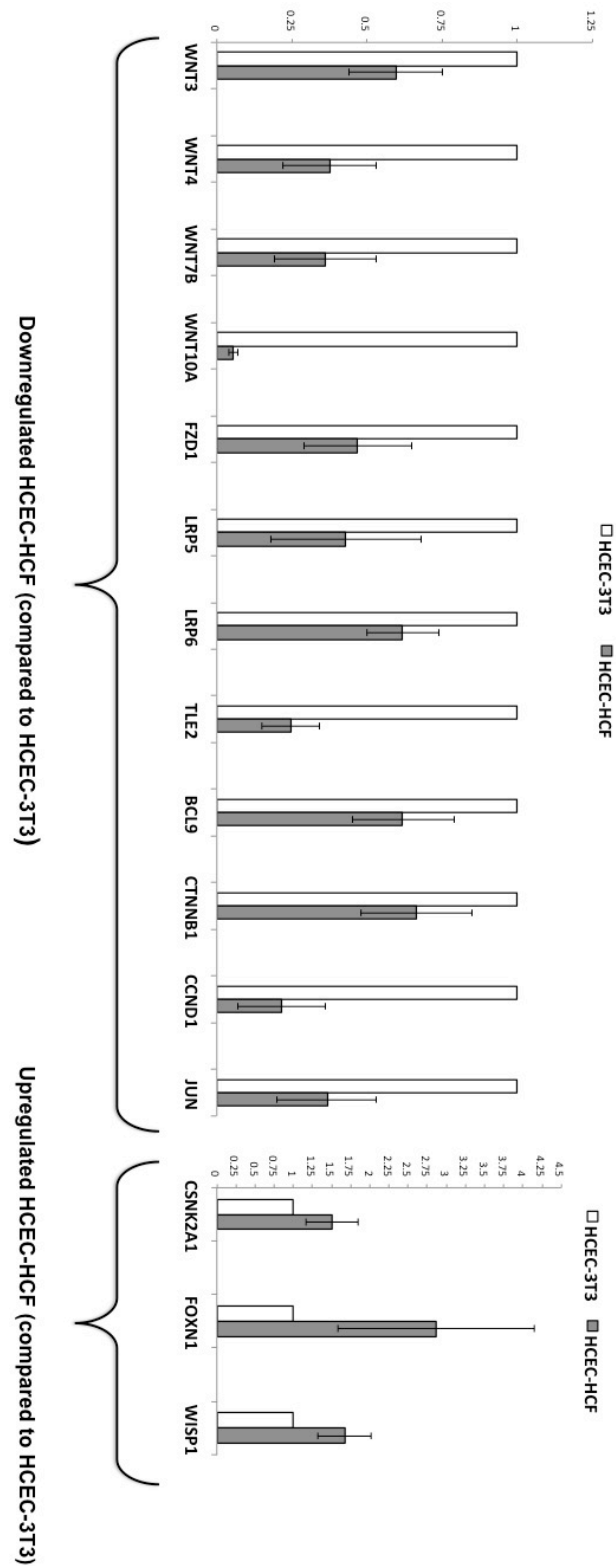


Figure 35: Up- and downregulation of genes with a significant difference in gene expression ($p \leq 0.05$) and a fold change (FC) ≥ 1.5 .

The bar chart shows the extent of alteration in gene expression under HCEC-HCF compared to HCEC-3T3 conditions found by quantitative PCR.

6.3.2.1. Protein Expression of β -Catenin and Cyclin D1 (CCND1)

For protein analysis β -Catenin was chosen because it is the central protein of the wnt signaling pathway and was shown to be significantly downregulated under HCEC-HCF conditions by QPCR. Cyclin D1 was chosen for protein analysis as it is a well established Wnt target gene which expression is directly influenced by the activity of wnt signaling and it was also shown to be significantly downregulated under HCEC-HCF conditions by QPCR.

The immunocytochemistry showed strong expression of cell membrane bound β -Catenin under HCEC-HCF and HCEC-3T3 conditions. Also cytoplasmic β -Catenin was found in some cells under both conditions (Fig. 36 A+B, arrows) but no difference was observed between both culture conditions and this was confirmed by western blot (Fig. 36C).

Examination of Cyclin D1 expression by immunocytochemistry revealed fewer Cyclin D1 positive epithelial cells and weaker staining under HCEC-HCF conditions compared to HCEC-3T3 conditions (Fig. 36D+E). This was confirmed by western blot experiments where weaker expression under HCEC-HCF conditions was found compared to HCEC-3T3 conditions (Fig. 33F).

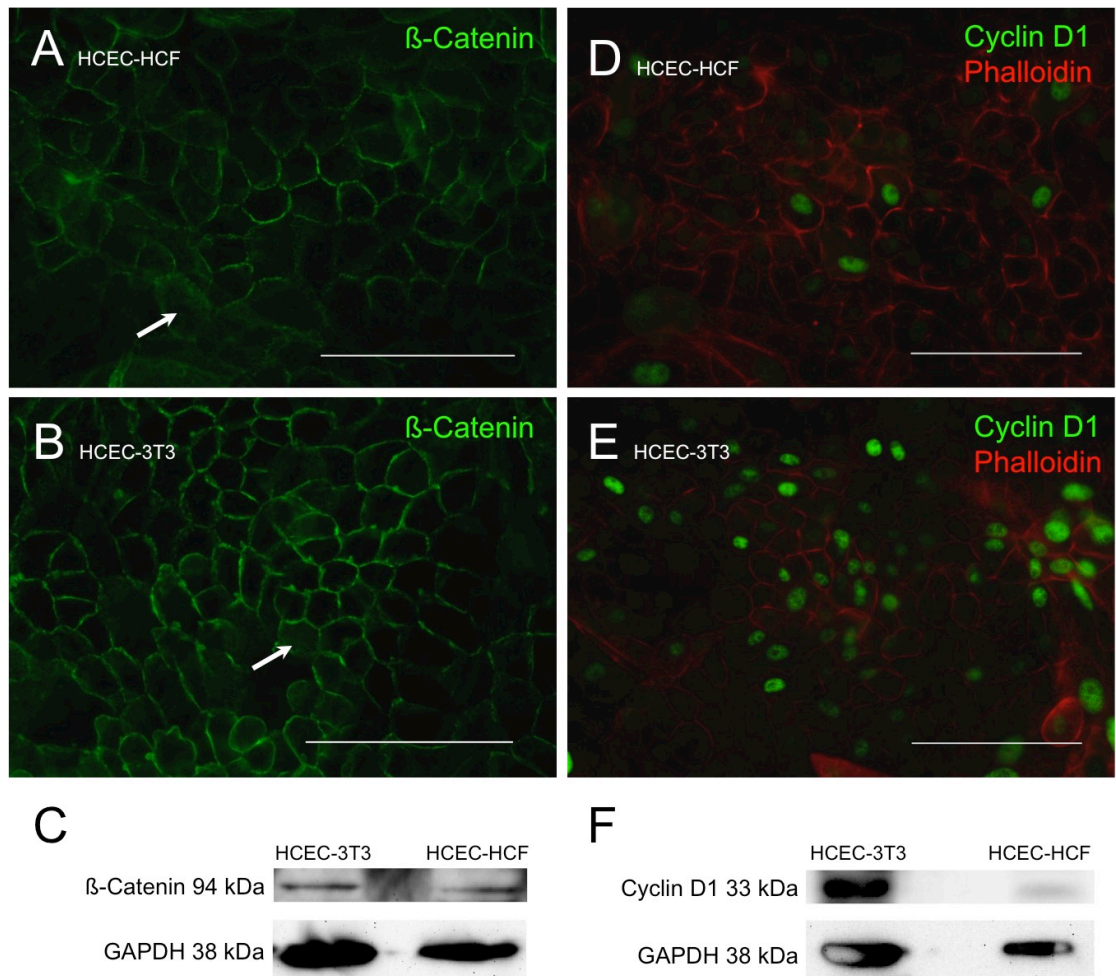


Figure 36: Protein Expression of β -Catenin and Cyclin D1 (CCND1) under HCEC-HCF and HCEC-3T3 conditions.

A-C: Immunocytochemistry showing strong expression of cell membrane bound β -Catenin (green) under HCEC-HCF (A) and HCEC-3T3 (B) conditions. Also cytoplasmatic β -Catenin was found in some cells under both conditions (A+B, arrows). No difference was observed between culture conditions and this was confirmed by western blot (C). **D-F:** Cyclin D1 (green) expression by immunocytochemistry revealed fewer Cyclin D1 positive epithelial cells and weaker staining under HCEC-HCF (D) compared to HCEC-3T3 (E) conditions. This was confirmed by western blot experiments (F) were weaker expression under HCEC-HCF conditions was found compared to HCEC-3T3 conditions. The cultures were counterstained phalloidin-TRITC (red, D+E). Scale bars, 100 μ m.

6.4. Discussion

The expansion of human ocular surface epithelial cells from a small biopsy on substrates like amnion or fibrin in the laboratory and then transplanting this sheet back onto the ocular surface of the same patient or another recipient represents one of the earliest successes in regenerative medicine (Tsai, Li et al. 2000; Tan, Ang et al. 2004; Ang, Tan et al. 2005; Rama, Matuska et al. 2010; Shortt, Tuft et al. 2010). However, one of the key questions of how to maintain and expand tissue specific stem or progenitor cells *in vitro* without causing differentiation still remains unanswered.

To identify important components of the niche environment, there is a need for *in vitro* models and recently our group has shown that mimicking of a “niche” environment *in vitro* by co-culturing ocular surface epithelial cells with mitotically active fibroblasts from the respective tissue in a complete serum free culture system supports the maintenance of epithelial progenitor cells (Notara, Shortt et al. 2010; Schrader, Notara et al. 2010).

In the present study we analysed the gene expression of conjunctival epithelial cells cultured in this *in vitro* niche model (HCEC-HCF) by microarray gene analysis, which revealed significant differences of gene expression in biological processes involved in cell proliferation, cell death, cell cycle, epithelium development and epithelial cell differentiation compared to control conditions (HCEC-3T3). Wnt pathway analysis by quantitative PCR revealed a downregulation of a number of genes under HCEC-HCF conditions strongly involved in the Wnt signaling pathway, including the Wnt ligands Wnt3, Wnt4,

Wnt7B, Wnt10A, Wnt receptor proteins FZD1, LRP5, LRP6, β -catenin, TCF7L1 and the important Wnt target gene Cyclin D1.

The Wnt signaling pathway describes a series of events that occur when Wnt proteins bind to cell-surface receptors of the frizzled family, ultimately resulting in an increased amount of β -catenin that reaches the nucleus inducing target gene expression (Nemeth, Topol et al. 2007). Wnt signaling has been strongly implicated in stem cell biology, but little is known about how stabilized β -catenin functions within the stem cell niche. Initially it was assumed, that Wnt proteins can act as stem cell growth factors, promoting the maintenance and proliferation of stem cells (Willert, Brown et al. 2003). However, more recent work suggests that activation of Wnt signaling on a low level supports stem cell maintenance, while medium activation causes stem cell activation and transition to transient amplifying cells, stronger activation leads to differentiation and excessive activation is associated with hyperproliferation, stem cell exhaustion and tumor formation (Lowry, Blanpain et al. 2005; Blanpain, Horsley et al. 2007; Castilho, Squarize et al. 2009; Gu, Watanabe et al. 2010).

β -Catenin, the central protein in the Wnt signaling pathway links E-cadherin and α -catenin to the cytoskeleton to form a complex that maintains normal epithelial polarity and intercellular adhesion. During Wnt activation β -catenin accumulates in the cytoplasm where it binds to cytosolic T cell-factor/lymphoid enhancer factor (LEF) transcription factors. The resulting complex is shuttled into the nucleus and activates the expression of target genes such as Cyclin D1 (Kato, Shimmura et al. 2007). Strong translocation of β -catenin to the

nucleus is not only observed in tumors (Norwood, Bailey et al. 2010), but also in hyperproliferative tissues, such as the pterygium in the conjunctiva (Kato, Shimmura et al. 2007). In our experiments we observed a slight (FC -1.51) but significant downregulation of β -catenin in the HCEC-HCF co-culture system compared to the HCEC-3T3 control. The immunocytochemistry showed a pattern of mainly membrane bound β -catenin under both conditions and also the western blot indicated no difference in β -catenin expression between the two conditions. However we observed a strong downregulation of the Wnt target gene Cyclin D1 under HCEC-HCF (FC -4.65) compared to the control condition and this was confirmed by immunocytochemistry and western blot.

It has been shown by Lowry et al. that activation of Wnt signaling in the hair follicle niche environment results in upregulation of cell cycle proteins, like Cyclin D1, which results in conversion of stem cells to proliferating transient amplifying cells. In that study Cyclin D1, a well established Wnt target gene was uniformly downregulated in the quiescent stem cell niche compartment as compared to their TA progeny (Lowry, Blanpain et al. 2005). This is consistent with our results, as we also found a reduction of Cyclin D1 in cells cultured under HCEC-HCF conditions and previous studies from our group have shown that the co-culture of mitotically active tissue specific fibroblasts with human epithelial cells in a serum free co-culture model (HCEC-HCF) improves the maintenance of epithelial cells with progenitor cell characteristics *in vitro* (Notara, Shortt et al. 2010; Schrader, Notara et al. 2010). The results from this study suggest that this is accompanied by a downregulation of several genes in the Wnt signaling pathway and Wnt target genes like Cyclin D1, which has

also been shown to be downregulated in the stem cell niche compartment *in vivo* (Lowry, Blanpain et al. 2005). It is therefore tempting to speculate that this *in vitro* co-culture model represents an environment that is more similar to the stem cell niche environment *in vivo* compared to the standard culture conditions. It also may suggest that controlled downregulation of Wnt signaling might improve the maintenance of epithelial cells with progenitor characteristics *in vitro* and that the reduced amount of Cyclin D1 positive cells in a cell population might be an indicator for that. However, more studies are needed to further investigate the involvement of Wnt signaling in the maintenance of epithelial cell with progenitor cell characteristics in this model by specific inhibition of these pathways and it would also be important to explore whether modifications of the environment like the addition of matrix components would further improve this *in vitro* niche system.

Chapter 7:

Exploration of amniotic membrane and compressed collagen gels as a carrier for *in vitro* expanded conjunctival epithelial cells

7. Exploration of amniotic membrane and compressed collagen gels as a carrier for *in vitro* expanded conjunctival epithelial cells

7.1. Introduction

Amniotic membrane has already been used for transplantation on the ocular surface (Honavar, Bansal et al. 2000; Barabino and Rolando 2003; Barabino, Rolando et al. 2003; Solomon, Espana et al. 2003; Henderson and Collin 2008) and it has also been shown to be a suitable substrate for the culture of conjunctival epithelial cells (Meller, Dabul et al. 2002; Tan, Ang et al. 2004; Ang, Tan et al. 2005). However, the results with amniotic membrane grafts for conjunctival reconstruction in the presence of chronic inflammatory ocular surface disease are not satisfactory (Solomon, Espana et al. 2003; Henderson and Collin 2008) as described in chapter 1.3.4. and 1.3.5. Other problems with the use of AM include reliable supply of membranes, costly screening, risk of disease transmission and considerable biological variations between donors (Levis, Brown et al. 2010).

Collagen meets many criteria for a transplantable substrate, it is biocompatible, has low immunogenicity, is naturally remodelled by cells and is relatively inexpensive to isolate (Levis, Brown et al. 2010). Also, the species source of the collagen is of limited clinical concern as implantation of animal collagen constructs (including the Food and Drug Administration (FDA) approved Apligraf) have been found to elicit little or no antigenic response in

patients (Falanga, Margolis et al. 1998; Brown, Wiseman et al. 2005). Unfortunately, collagen hydrogels are inherently weak due to their high water content, which make them less suitable for transplantation (Brown, Wiseman et al. 2005; Levis, Brown et al. 2010). However, in 2005 Brown et al., reported a new process for the controlled engineering of biomimetic scaffolds by rapid removal of fluid from hyperhydrated collagen gel constructs, using plastic compression (PC). In their study, PC fabrication produced dense, cellular, mechanically strong native collagen structures (Brown, Wiseman et al. 2005) and these structures have also been shown to be suitable for the culture of epithelial cells (Levis, Brown et al. 2010).

Therefore, aim of this experimental series was to explore the suitability of PC collagen as a substrate for conjunctival epithelial cells and compare it to AM.

7.2. Methods

The methods used for the experiments in this chapter are described in chapter 2 (Materials and Methods). Human amniotic membrane and human conjunctival tissue samples from three different donors were used. Conjunctival cells from three different donors were used for the cell culture experiments. Three biological repeats were conducted and for the force measurements three repeats were performed per tissue per experiment. The methods specific to this chapter are described below.

7.2.1. Preparation of Human Amniotic Membrane

Human tissue was handled according to the Tenets of the Declaration of Helsinki. Following informed consent of the donor, human amniotic membrane (AM) was obtained at the time of elective caesarean section. Under sterile conditions, intact amnion was stripped from the chorion by blunt dissection (Fig. 37A+B) and sutured onto 7.5 x 7.5 cm sterile sponge sheets (Katena®) with the epithelial side facing up (Fig. 37C). The membranes were cryopreserved at -80°C in Dulbecco's modified Eagle's medium (Fig. 37D). Directly before use the amniotic membrane was incubated with TrypLE™ Select 1x (GIBCO) for 30 minutes and after that the remaining amniotic epithelial cells were removed by gentle scraping using a cell scraper.

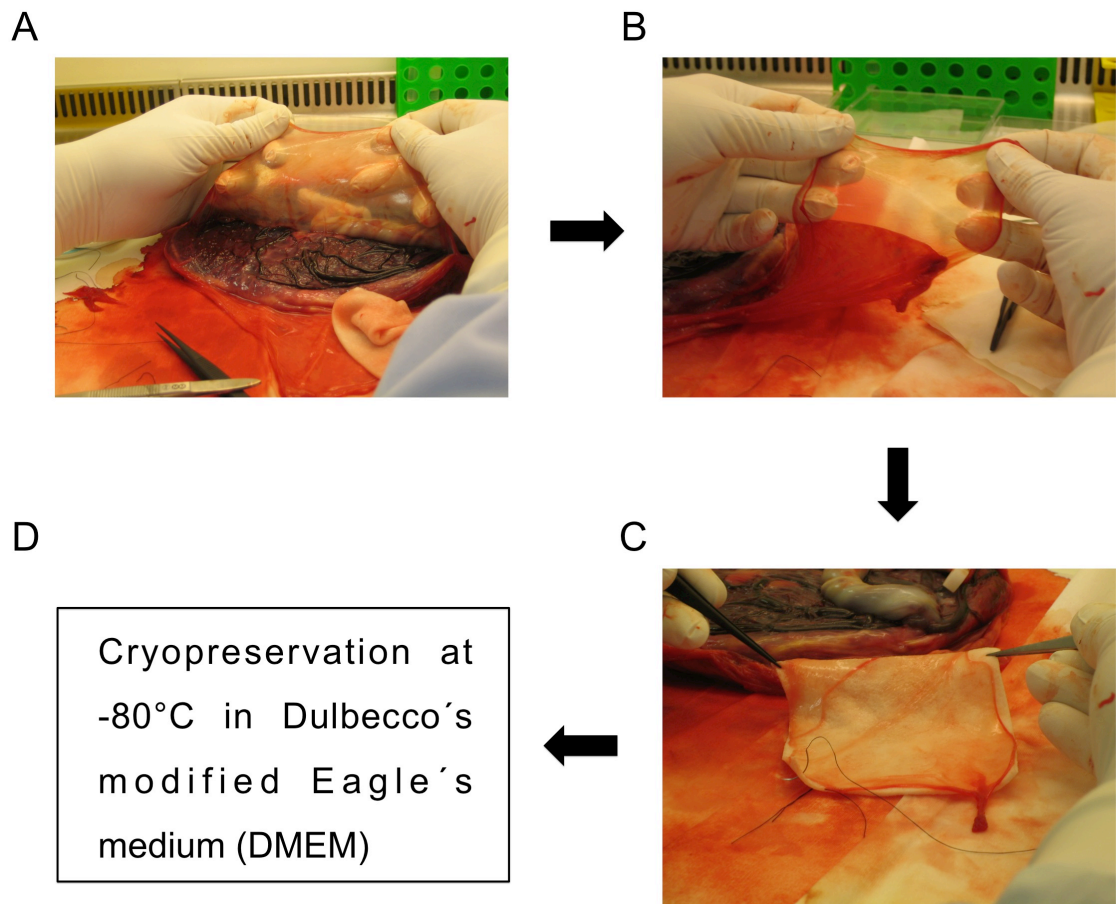


Figure 37: Preparation of the amniotic membrane

7.2.2. Preparation of the plastic compressed collagen gel matrices

Plastic compressed (PC) collagen gels were prepared as described before (Brown, Wiseman et al. 2005; Levis, Brown et al. 2010). For the preparation of four collagen gels, 8 ml of sterile rat-tail type I collagen (2.06 mg ml⁻¹; First Link, Birmingham, UK) and 1 ml of Minimum Essential Medium (Invitrogen Ltd, Paisley, UK) were mixed and neutralized using sodium hydroxide (Sigma-Aldrich, Dorset, UK). After that, 1 ml of medium containing 880.000 conjunctival fibroblasts (220.000 fibroblasts/gel) was added or 1 ml of medium alone for the casting of acellular gels and the solution was left on ice for 30

minutes before casting to prevent gelling while allowing dispersion of any small bubbles within the solution. The gels were cast, by filling the solution in the wells of a 12-well plate (2.5 ml/well), which was then placed in a 5% CO₂ incubator at 37 °C for 30 minutes to allow the gels to undergo fibrillogenesis. A nylon mesh and a chromatography paper disc were then placed on the gels, followed by a roll of approximately one meter chromatography paper. The gels were then subjected to unconfined compression by applying a weight of 35 g for 15 minutes. After removal of the weight, filter paper and nylon mesh, the collagen gel constructs were covered with culture medium until the addition of epithelial cells (Fig. 38).

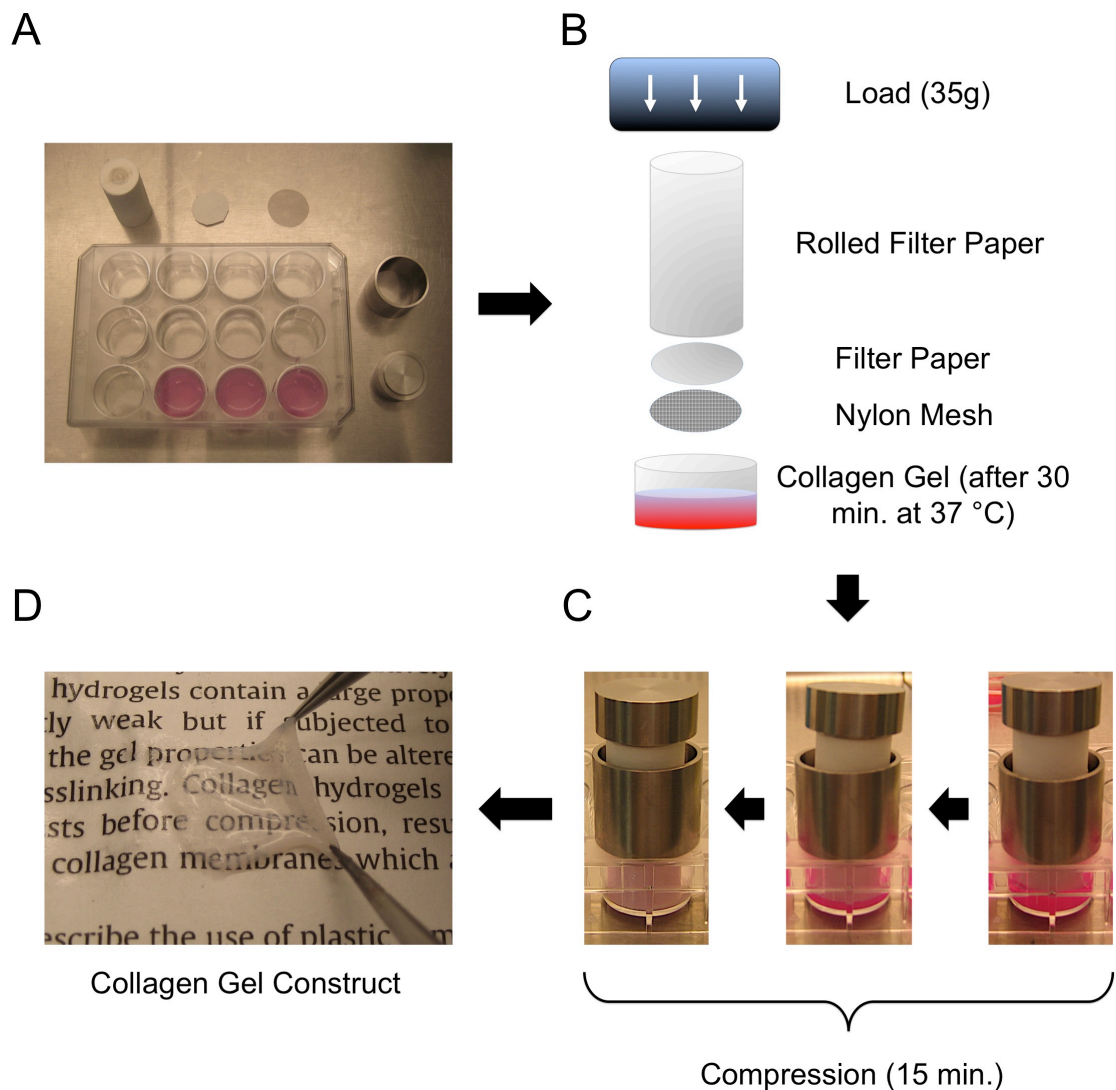


Figure 38: Process of plastic compression.

The gels were cast, by filling the neutralized collagen solution in the wells of a 12-well plate, which was then placed in a 5% CO₂ incubator at 37 °C for 30 minutes to allow the gels to undergo fibrillogenesis (A). A nylon mesh and a chromatography paper disc were then placed on the gels, followed by a roll of chromatography paper (B). The gels were then subjected to unconfined compression by applying a weight of 35 g for 15 minutes (C). D: Collagen gel construct after plastic compression.

7.2.3. Break strength tests

The break strength measurements were performed by Dr. Alvena Kureshi, who is very experienced in mechanical testing of tissues and who worked as a collaborator on this part of the project. The PC collagen sheets, the amnion and the conjunctiva were cut into 'dog-bone' shaped strips measuring 2-5 mm width at the central section and 10 mm length using a scalpel blade. The shaping of specimens was important as this allows maximum stress to be placed on the centre of the specimen, rather than on the anchor points, ensuring fracture would occur within the material bulk. Metal mesh grips were attached to each end of the strip to ensure secure anchorage of the specimen in the tensile strength testing device (*in vitro* culture test rig) (Fig. 39) (Afoke, Meagher et al. 1998). Each sample was anchored in metal clamps at the lower end of the creep device and attached to a pivoting bar at the upper end (Fig. 39A). The glass chamber (Aimer Products Ltd, UK) was filled with PBS to prevent dehydration of the sample. A series of weights (Fig. 39B) were applied to a single tissue strip anchored in this manner, until it broke. Only samples that broke in the middle were included. Three samples were used per condition and the area of the cross section of the stripes were calculated by multiplication of the width and the thickness of the strips. The thickness was determined by measuring the thickness of paraffin sections of the respective tissues. The break stress was calculated from the force (N) divided by the area of the cross section of the tissue strip (m^2) (Formula: break stress=force/area).

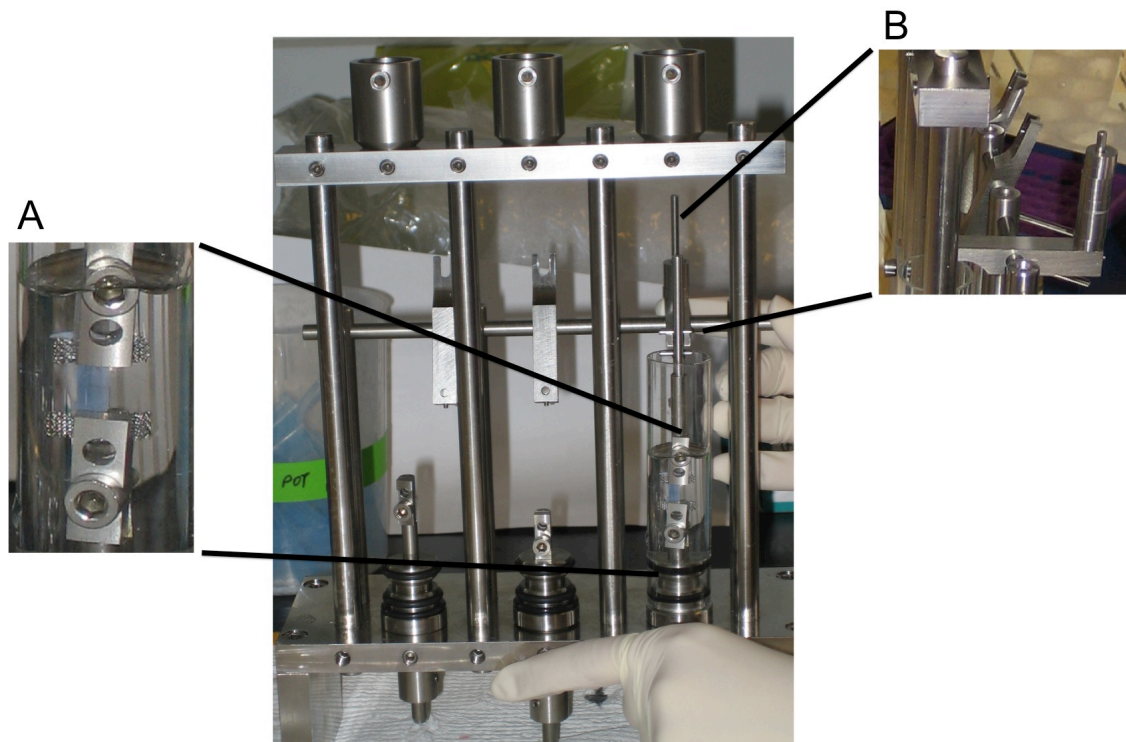


Figure 39: Break strength testing device.

The samples were anchored in metal clamps at the lower end of the creep device and attached to a pivoting bar at the upper end (A). The glass chamber was filled with PBS to prevent dehydration of the sample. A series of weights (B) were applied to a single tissue strip anchored in this manner, until it broke.

7.3. Results

7.3.1. Denuding of the amniotic membrane

To ensure that all the amniotic epithelial cells were removed from the basement membrane of the AM before the seeding of the conjunctival epithelial cells, a small sample of the AM was taken before and after the denuding procedure and stained with trypan blue (0.4%, Gibeco). The staining with trypan blue revealed a sheet of amniotic epithelial cells on the AM before

(Fig. 40A) and almost no epithelial cells on top of the AM after the denudation procedure (Fig 40B).

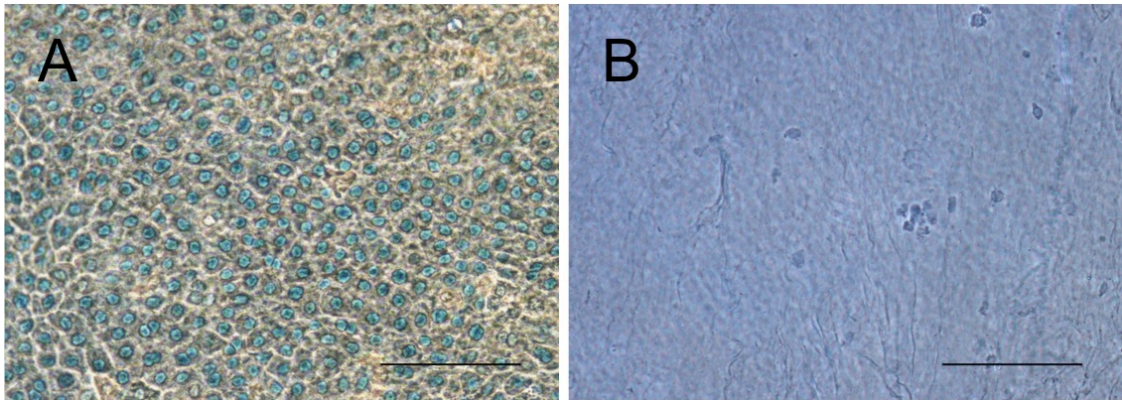


Figure 40: Denudation procedure of the AM.

A: AM before denudation, showing a sheet of amniotic epithelial cells stained by trypan blue. B: AM after denudation, almost completely free of amniotic epithelial cells. Scale bars, 100 μm .

7.3.2. Evaluation of different conjunctival epithelial cell densities on PC collagen gels and AM

To determine the appropriate seeding density for the conjunctival epithelial cells, a dilution series was performed, where 1×10^5 (318 cells/ mm^2), 3×10^5 (955 cells/ mm^2) and 6.5×10^5 (2069 cells/ mm^2) epithelial cells were seeded on PC collagen gels and compared to AM.

When 318 cells/ mm^2 were seeded on the PC collagen gels and the AM, after one week of culture, a single layer of epithelial cells was found on top of the matrices. However the epithelial layer was incomplete and the epithelial cells mostly had a flattened appearance on both, gels (Fig. 41A+B) and AM (Fig. 42A+B).

When 955 cells/mm² and 2069 cells/mm² were seeded, a complete layer of mostly small and morphologically undifferentiated epithelial cells were observed on the PC collagen gels and the AM after one week of culture (Fig. 41 and 42 C+D and E+F). Also multilayering was found on both matrices and this seemed to be more pronounced on the PC collagen gels and the AM were 2069 cells/mm² were seeded (Fig 41 and 42 E+F).

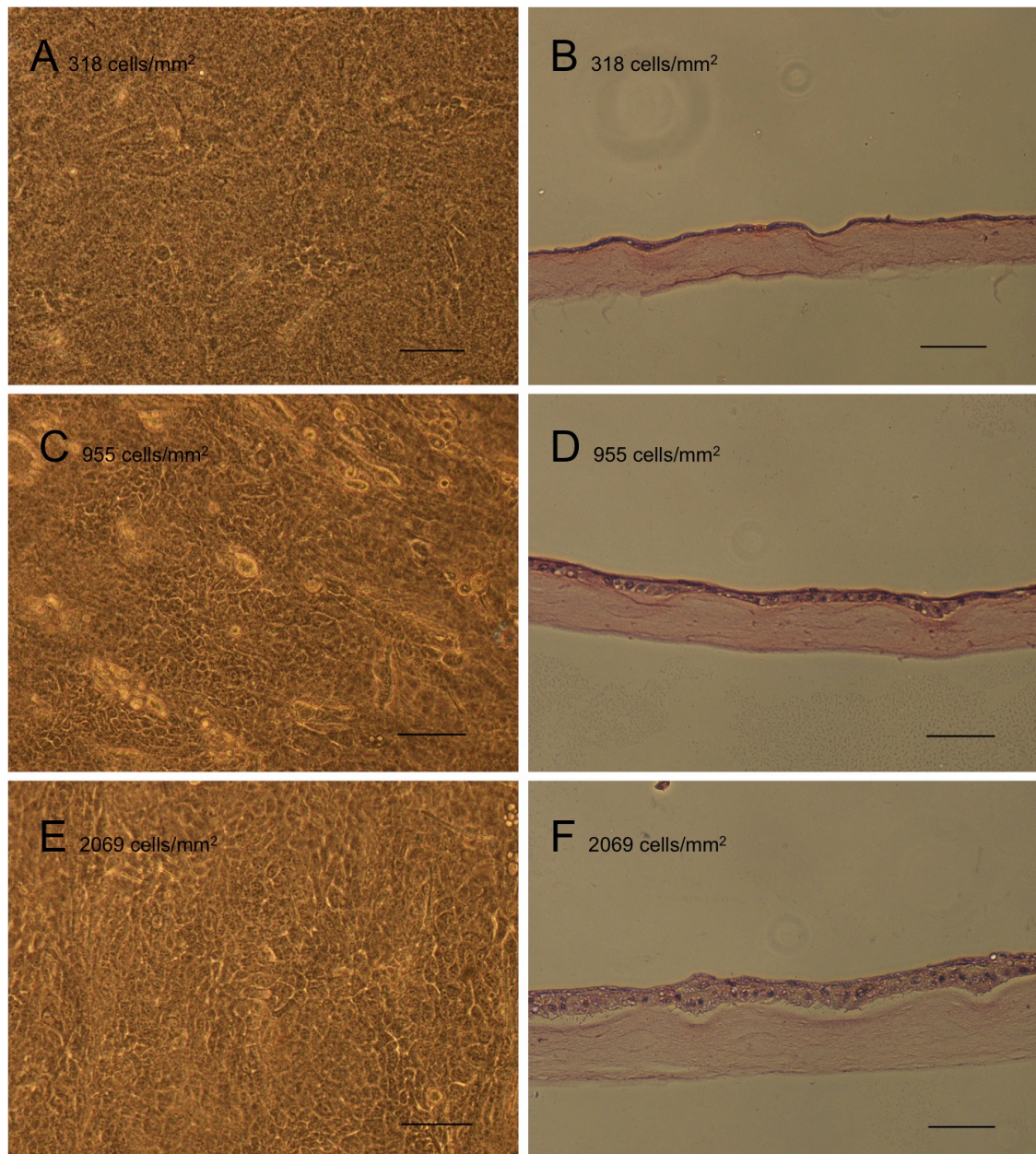


Figure 41: Evaluation of different seeding densities of epithelial cells on PC collagen gels after one week of submerged culture. Scale bars, 100 μm .

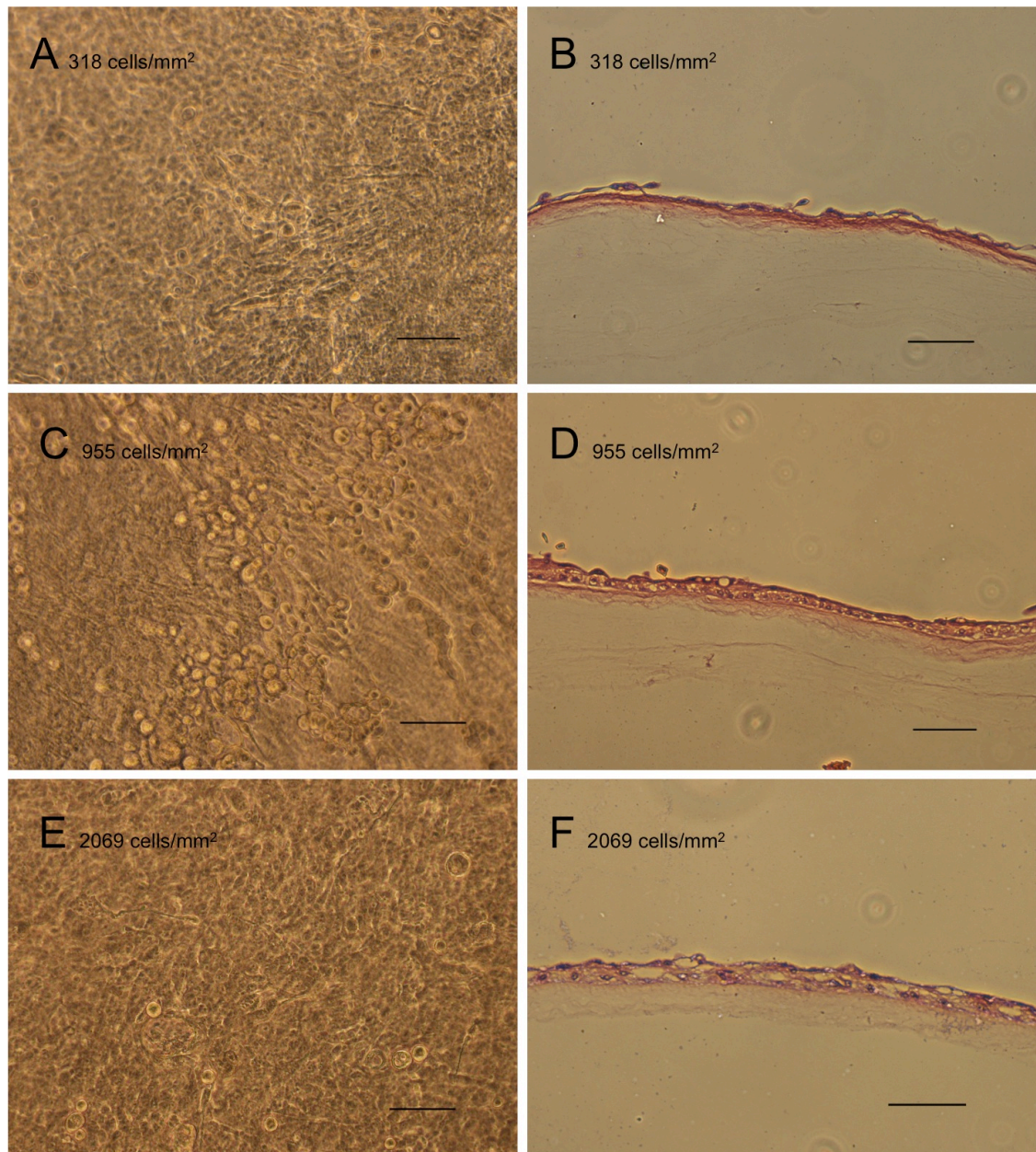


Figure 42: Evaluation of different seeding densities of epithelial cells on AM after one week of submerged culture. Scale bars, 100 µm.

7.3.2.1. Comparison of PC collagen gels with and without fibroblasts and AM as carriers for conjunctival epithelial cells

To determine whether PC collagen gels containing conjunctival fibroblasts were suitable as carriers for conjunctival epithelial cells, 2069 epithelial

cells/mm² were seeded on PC collagen gels with and without conjunctival fibroblasts and AM.

After one week of submerged culture a complete layer of mostly small and morphologically undifferentiated epithelial cells were observed on the AM (Fig. 43A-C) as well as on the PC collagen gels without (Fig. 43D-F) and with conjunctival fibroblasts (arrowheads) (Fig. 43G-I). Also, the beginning of multilayering was observed on parts of all three matrices.

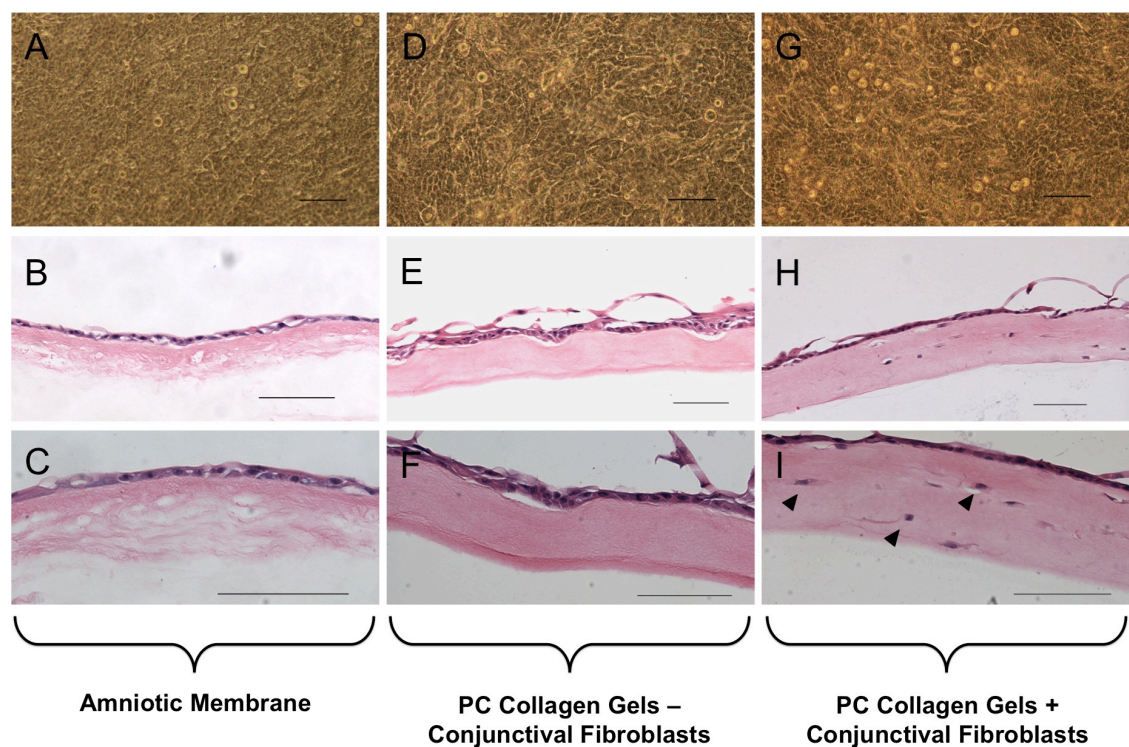


Figure 43: Comparison of PC collagen gels with and without fibroblasts and AM as carriers for conjunctival epithelial cells. Scale bars, 100 μ m.

7.3.2.2. Break strength tests

Preliminary tests of the biomechanical properties of the different matrices were performed using a tensile strength testing device. Hereby the break strength of undenuded and denuded AM as well as PC collagen gels with and without fibroblasts was tested and compared to human conjunctiva. Also the effect of a culture period with conjunctival epithelial cells for one week on the break strength was evaluated.

The one-way Analysis Of Variance (ANOVA) revealed a significant difference between the groups ($p \leq 0.0004$). The Bonferroni post-hoc test between the groups showed no significant difference in the break strength before and after denudation of the AM. Also no significant difference in break strength was found between human conjunctiva and PC collagen gels with and without fibroblasts and also a culture period of one week did not have any significant effect on the break strength of the PC collagen gels and the AM. However untreated AM and AM after one week of culture showed a significantly higher break strength compared to the PC collagen gels and the human conjunctiva (Fig. 44).

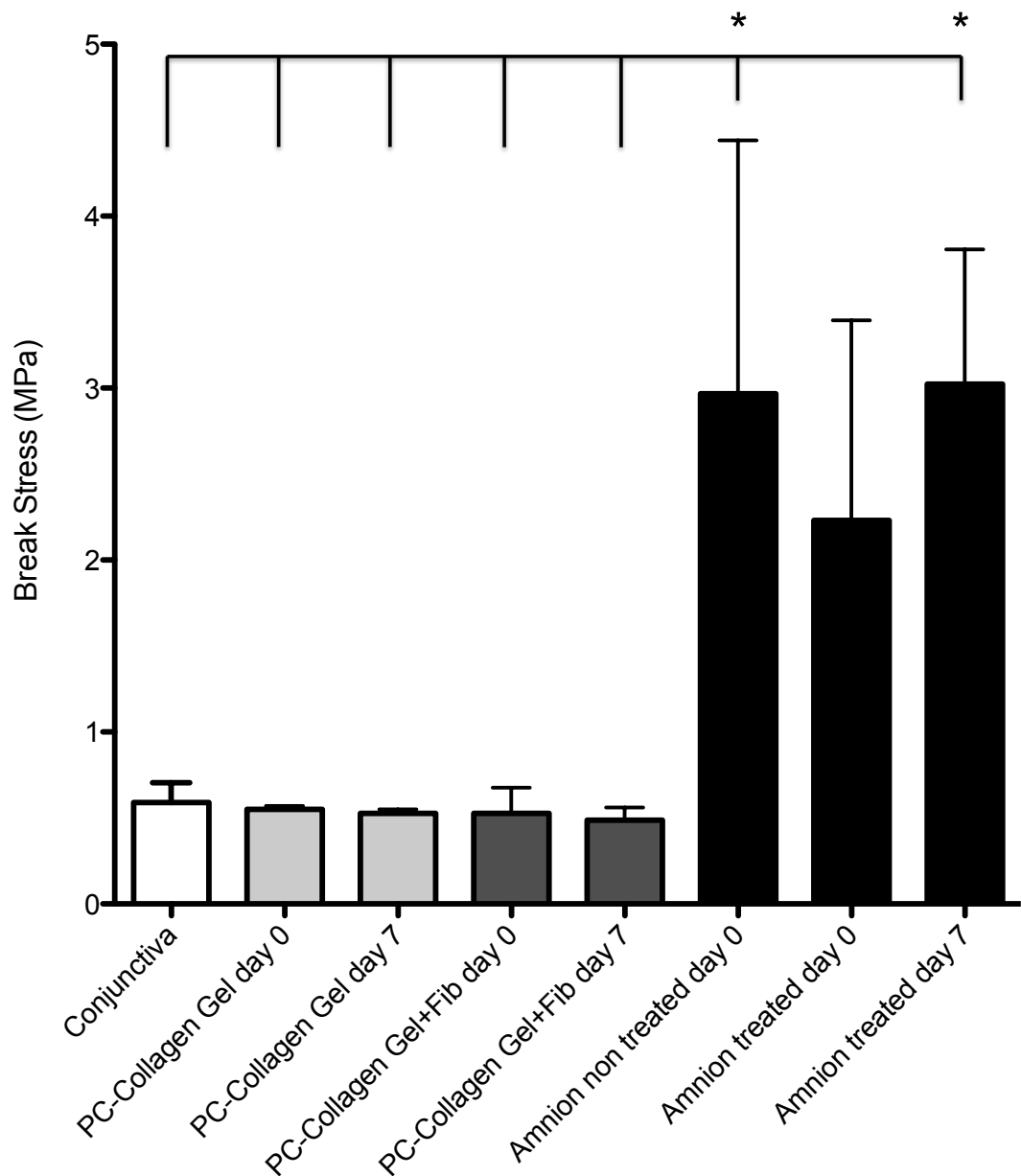


Figure 44: Break strength tests of pc collagen gels, AM and human conjunctiva.

No significant difference in break strength before and after denudation of the AM was found. Also, no significant difference in break strength was found between human conjunctiva and PC collagen gels with and without fibroblasts. Also the culture period did not have any significant effect on the break strength of the PC collagen gels and the AM. However untreated AM and AM after one week of culture showed a significantly

higher break strength compared to PC collagen gels and human conjunctiva.

7.4. Discussion

In this experimental series it was demonstrated that PC collagen gels as well as amniotic membrane can be successfully used as substrates for the cultivation of conjunctival epithelial cells. Furthermore conjunctival fibroblasts can be successfully seeded into the PC collagen gels, resulting in a matrix cell compound more similar to human conjunctiva *in vivo*. Comparison of the break stress between the tissues revealed that the AM was substantially stronger in terms of break stress compared to the PC collagen gels and human conjunctiva, but also the variation between the different donor AM's was very high resulting in relatively high standard deviations compared to a very low variability between the PC collagen gels. The break stress values of the PC collagen gels were very similar to the human conjunctiva indicating similar strength of these tissues. However, these measurements were preliminary and more defined measurements using a more sophisticated mechanical testing device that can measure dynamic stress-strain values have to follow.

In conclusion, according to these preliminary experiments, PC collagen gels without and with conjunctival fibroblasts can be successfully used as a substrate for conjunctival epithelial cells and might be an alternative to amniotic membrane for reconstruction of the conjunctiva. However more work is required to investigate the potential of the PC collagen gels to serve as a niche structure with the ability to preserve conjunctival progenitor cells. It also

needs further experiments to evaluate the potential benefits of seeding conjunctival fibroblasts into the collagen substrates in terms of cross talk with the epithelial cells, remodeling of the matrix and progenitor cell preservation. Moreover the mechanical properties need to be further explored and also safety and efficacy testing in an in vivo model will be required to make sure that these constructs are safe for the use in humans.

Chapter 8:

General Discussion

8. General Discussion

Reconstruction of the conjunctiva is an essential part of ocular surface regeneration, especially if an extensive area or the whole ocular surface is affected, such as in patients with ocular cicatricial pemphigoid, Stevens-Johnson syndrome or chemical/thermal burns (Barabino, Rolando et al. 2003; Espana, Di Pascuale et al. 2004; Tseng, Di Pascuale et al. 2005). However, there is a lack of suitable donor tissue for conjunctival replacement, especially when large grafts are required and it is important that new materials and methods are developed for conjunctival reconstruction. The aims of this thesis were to characterise the conjunctival epithelial cell population and to improve the maintenance of the epithelial progenitor cells during *in vitro* expansion in order to make advances towards producing conjunctival epithelial cells suitable for therapeutic use. The final aim was to transfer these cells to compressed collagen matrices and amniotic membrane and test the properties of these cell-matrix constructs.

Different methods using either single cell suspensions (Pellegrini, Golisano et al. 1999) or conjunctival tissue explants (Diebold, Calonge et al. 1997; Risse Marsh, Massaro-Giordano et al. 2002; Ang, Tan et al. 2004) have been described for the cultivation of conjunctival epithelial cells *in vitro*. The use of the “explant method” offers the advantage that only a very small biopsy is needed for the expansion of the conjunctival epithelial cells, which is more suitable in a clinical setting, where biopsies are obtained from patients for *in*

vitro epithelial cell expansion and transplantation and the amount of tissue that can be biopsied is very limited.

When epithelial cells are expanded *in vitro* from small biopsies it is also important to know whether cells with progenitor cell characteristics migrate from their “niche” environment out of the biopsy onto the culture dish, as progenitor cells are needed to maintain the conjunctival epithelial cell population on a conjunctival substitute suitable for transplantation (Pellegrini, Golisano et al. 1999; Henderson and Collin 2008). There is evidence that epithelial progenitor cells can migrate out of an explanted biopsy (Li, Hayashida et al. 2007). However, it has also been shown that stem cell properties from epithelial cells decrease with the distance from the original explant and that the proximity of the stem cell population to their original niche environment in the explant might be important in maintaining the stem cell population in an undifferentiated stage (Kolli, Lako et al. 2008). Our experiments showed that conjunctival epithelial cells grown out of explants exhibited progenitor cell characteristics, such as a high nuclear to cytoplasmic ratio, indicating a still undifferentiated stage and a strong expression of the putative progenitor cell markers p63 α , ABCG2 and CK15. Additionally the epithelial cells demonstrated a high total colony forming efficiency and a high percentage of colonies with the characteristics of holoclone colonies (Barrandon and Green 1987). Also, we were able to demonstrate, that epithelial cells grown out of the explants were capable to produce holoclone-, meroclone- and paraclone- colonies. Holoclones are supposed to be generated by stem cells (Barrandon and Green 1987; Pellegrini, Golisano et

al. 1999) and the generation of holoclone colonies from cells grown out of explant cultures give evidence that progenitor cells are migrating from the explants onto the culture dish.

In conclusion these experiments showed that conjunctival epithelial cells can be successfully expanded from biopsies using an explant culture method and that this epithelial cell population contains a subpopulation of cells which shows characteristics of epithelial progenitor cells, which are essential for the maintenance of the epithelial cell population on a conjunctival substitute aimed for transplantation to the ocular surface.

A major problem for the clinical application of cell based therapies, such as the transplantation of epithelialised cell sheets, is the shortage of donor tissue and ensuring an epithelialised graft is available when a patient needs it. The use of cryopreserved conjunctival epithelial cells could significantly facilitate the delivery of grafts from the laboratory to the clinic. As cells with stem cell characteristics are needed to ensure long-term epithelial function in-vivo after transplantation (Pellegrini, Golisano et al. 1999; Henderson and Collin 2008), we investigated the effect of cryopreservation and long-term culture on the progenitor cell characteristics of conjunctival epithelial cells. The results of this chapter showed that cryopreservation seems to have no effect on the colony forming efficiency and the proliferative potential of the cells, as no significant difference was found in these parameters before and after cryopreservation. Additionally, a cryopreservation duration of more than 6 months seems not to alter the progenitor cell characteristics of conjunctival epithelial cells as no

difference was observed in cell viability, immunoreactivity to p63 α and ABCG2 and colony forming capacity between 14 days and 202.7 ± 13.0 days of cryopreservation. However it is possible that the cryopreservation process affects other parameters, which were not included in this study.

In conclusion cryopreservation with 10% DMSO supplemented with 20% FBS was effective in preserving conjunctival epithelial cells with progenitor cell characteristics and it had no detectable effect on the progenitor cell marker expression the colony forming efficiency and the proliferative capacity during long term in-vitro expansion. The use of cryopreserved conjunctival epithelial cells could improve the supply of tissue for cell therapy applications in the clinic.

In any conjunctival substitute a conjunctival stem cell population must be present to ensure the survival of the epithelial layer on the reconstructed ocular surface (Pellegrini, Golisano et al. 1999; Henderson and Collin 2008; Schrader, Notara et al. 2009) and stem cells obtained from a small biopsy of normal conjunctiva must be expanded *in vitro* before transplantation. Stem cells are regulated by intercellular interactions, the external environment and the underlying mesenchyme forming microenvironments known as stem cell niches (Revoltella, Papini et al. 2007) and it has been shown that these tissue specific niche environments are essential for stem cell maintenance and the control of the epithelial cell-fate determination (Spradling, Drummond-Barbosa et al. 2001; Blanpain, Horsley et al. 2007).

In this study, conjunctival epithelial cells co-cultured with conjunctival fibroblasts in this serum free co-culture model, showed a high expression of the putative progenitor cell markers p63 α and ABCG2. The colony forming efficiency assays showed that cells cultured under HCEC-HCF conditions were able to produce smooth colonies containing small and tightly packed cells which had a significant higher total colony forming capacity compared to cells cultured under HCEC-3T3 conditions. Also the number of colonies with a surface area > 10mm², referred to as holoclone colonies (Barrandon and Green 1987) were significantly higher in the HCEC-HCF group compared to the HCEC-3T3 group indicating that this artificial niche environment (HCEC-HCF) is superior in maintaining a progenitor cell population compared to the standard co-culture conditions (HCEC-3T3).

Previous studies have suggested the presence of a bipotent conjunctival precursor cell that is able to give rise to both non-goblet and goblet cells (Wei, Lin et al. 1997; Pellegrini, Golisano et al. 1999) and that the commitment to differentiate into goblet cells occurs relatively late, so that goblet cells are preferentially generated by more differentiated transient amplifying cells (Pellegrini, Golisano et al. 1999). In the experiments conducted for this thesis single cells and groups of MUC5AC positive and PAS positive cells under HCEC-3T3 culture conditions were found, whereas under HCEC-HCF conditions these cells were only very rarely found. As goblet cell differentiation is supposed to occur preferentially in more differentiated transient amplifying cells, these results indicate that the HCEC-HCF condition may be superior in

maintaining a more undifferentiated cell population compared to the HCEC-3T3 condition.

In conclusion, mimicking of a simple “niche” environment *in vitro* by co-culturing mitotically active conjunctival fibroblasts with conjunctival epithelial cells supports the maintenance of conjunctival cells with progenitor cell characteristics and therefore might be a useful tool to expand conjunctival epithelial cells with progenitor cell characteristics *in vitro* for clinical use and to investigate cell-cell interactions between fibroblasts and epithelial cells in an *in vitro* niche environment model.

To identify important components of the niche environment *in vitro* niche models may be very important. It was shown here that mimicking a niche environment *in vitro* by co-culturing ocular surface epithelial cells with mitotically active fibroblasts from the respective tissue in a complete serum free culture system supports the maintenance of epithelial progenitor cells (Notara, Shortt et al. 2010; Schrader, Notara et al. 2010).

To examine the underlying mechanisms of this improved maintenance of epithelial progenitor cells, we analysed the gene expression of conjunctival epithelial cells cultured in this *in vitro* niche model (HCEC-HCF) by microarray gene analysis, which revealed significant differences of gene expression in biological processes involved in cell proliferation, cell death, cell cycle, epithelium development and epithelial cell differentiation compared to control conditions (HCEC-3T3). Wnt pathway signaling analysed by quantitative PCR revealed downregulation of a number of genes under HCEC-HCF conditions

strongly involved in the Wnt signaling pathway, including the Wnt ligands Wnt3, Wnt4, Wnt7B, Wnt10A, Wnt receptor proteins FZD1, LRP5, LRP6, β -catenin, TCF7L1 and the important Wnt target gene Cyclin D1.

β -Catenin, the central protein in the Wnt signaling pathway links E-cadherin and α -catenin to the cytoskeleton to form a complex that maintains normal epithelial polarity and intercellular adhesion. During Wnt activation β -catenin accumulates in the cytoplasm where it binds to cytosolic T cell-factor/lymphoid enhancer factor (LEF) transcription factors. The resulting complex is shuttled into the nucleus and activates the expression of target genes such as Cyclin D1 (Kato, Shimmura et al. 2007). Strong translocation of β -catenin to the nucleus is not only observed in tumors (Norwood, Bailey et al. 2010), but also in hyperproliferative tissues, such as the pterygium in the conjunctiva (Kato, Shimmura et al. 2007). In our experiments a slight (FC -1.51) but significant downregulation of β -catenin in the HCEC-HCF co-culture system compared to the HCEC-3T3 control was observed. Immunocytochemistry showed a pattern of mainly membrane bound β -catenin under both conditions and also the western blot indicated no difference in β -catenin expression between the two conditions. However a strong downregulation of the Wnt target gene Cyclin D1 under HCEC-HCF (FC -4.65) was observed compared to the control condition and this was confirmed by immunocytochemistry and western blot.

It has been shown by Lowry et al. that activation of Wnt signaling in the hair follicle niche environment results in upregulation of cell cycle proteins, such as Cyclin D1, which results in conversion of stem cells to proliferating transient amplifying cells. In that study Cyclin D1, a well established Wnt target gene

was uniformly downregulated in the quiescent stem cell niche compartment as compared to their TA progeny (Lowry, Blanpain et al. 2005). This is consistent with our results, where a reduction of Cyclin D1 in cells cultured under HCEC-HCF conditions and as described in chapter 3.3., was also found. Previous experiments from our group have shown that the co-culture of mitotically active tissue specific fibroblasts with human epithelial cells in a serum free co-culture model (HCEC-HCF) improves the maintenance of epithelial cells with progenitor cell characteristics *in vitro* (Notara, Shortt et al. 2010; Schrader, Notara et al. 2010). The results from this study suggest that this is accompanied by a downregulation of several genes in the Wnt signaling pathway and Wnt target genes like Cyclin D1, which has also been shown to be downregulated in the stem cell niche compartment *in vivo* (Lowry, Blanpain et al. 2005). It is therefore tempting to speculate that this *in vitro* co-culture model represents an environment that is more similar to the stem cell niche environment *in vivo* compared to the standard culture conditions. It also may suggest that controlled downregulation of Wnt signaling might improve the maintenance of epithelial cells with progenitor characteristics *in vitro* and that the reduced amount of Cyclin D1 positive cells in a cell population might be an indicator for that.

In the last results chapter of this thesis, the feasibility of amniotic membrane and compressed collagen gels as a carrier for *in vitro* expanded conjunctival epithelial cells was explored. In this experimental series it was demonstrated that PC collagen gels as well as amniotic membrane can be successfully used

as substrates for the cultivation of conjunctival epithelial cells. Furthermore, conjunctival fibroblasts can be successfully seeded into the PC collagen gels resulting in a matrix cell compound more similar to human conjunctiva in vivo. Comparison of the break stress between the tissues revealed that the AM was substantially stronger in terms of break stress compared to the PC collagen gels and human conjunctiva, but also the variation between the different donor AM's was very high resulting in relatively high standard deviations compared to a very low variability between the PC collagen gels. The break stress values of the PC collagen gels were very similar to the human conjunctiva indicating similar strength of these tissues. However, these measurements were preliminary and more defined measurements using a more sophisticated mechanical testing device that can measure dynamic stress-strain values have to follow.

In conclusion, according to these preliminary experiments, PC collagen gels without and with conjunctival fibroblasts can be successfully used as a substrate for conjunctival epithelial cells and PC collagen based epithelial cell sheets might be an alternative to amniotic membrane for reconstruction of the conjunctiva.

8.1. Conclusion

In conclusion the work leading to this thesis clarified several aspects important for the development of a conjunctival substitute suitable for clinical applications. First, that the conjunctival epithelial cell population grown out of conjunctival tissue explants contain a population of cells with progenitor cell

characteristics which are essential to maintain the cell population on a conjunctival tissue substitute. Second, that cryopreservation with DMSO supplemented with 20% FBS is effective in preserving conjunctival epithelial cells with progenitor cell characteristics, which could improve the supply of conjunctival epithelial cells for cell therapy applications. Third, that mimicking a “niche” environment *in vitro* by co-culturing mitotically active conjunctival fibroblasts with conjunctival epithelial cells is accompanied by a downregulation of genes involved in the Wnt signaling pathway and supports the maintenance of conjunctival cells with progenitor cell characteristics. Therefore this might be a useful tool to expand conjunctival epithelial cells with progenitor cell characteristics *in vitro* for clinical use. Fourth, that PC collagen gels without and with conjunctival fibroblasts can be successfully used as a substrate for conjunctival epithelial cells and PC collagen based epithelial cell sheets might be an alternative to amniotic membrane for reconstruction of the conjunctiva.

Future Work

9. Future Work

The ultimate goal of the project is to obtain a stable conjunctival substitute for conjunctival reconstruction, which then can be tested in a clinical trial and future work will need to focus on different aspects.

9.1. Improvement of the culture conditions for conjunctival epithelial cells in order to maintain the stem cell population during cell expansion *in vitro*

In vitro expansion of conjunctival epithelial cells results in a decrease of epithelial cells with progenitor characteristics in the culture over time (Tsai, Ho et al. 1994; Schrader, Notara et al. 2009). However in any conjunctival substitute a conjunctival stem cell population must be present to ensure the survival of the epithelial layer on the reconstructed ocular surface (Pellegrini, Golisano et al. 1999; Henderson and Collin 2008) and a recent study by Rama et al. indicated that a defined percentage of epithelial stem cells is necessary (however not sufficient) to ensure a good clinical result in limbal stem cell grafts (Rama, Matuska et al. 2010).

Also, standard culture systems for epithelial cells still depend on animal derived products like foetal calf serum and also on the use of growth arrested 3T3 feeder cells. Different serum free and animal product free culture systems have been tested for the expansion of ocular surface epithelial cells, but the co-culture with growth arrested 3T3 feeder cells in the presence of foetal calf serum containing medium seems to be most suitable at present (Ang, Tan et

al. 2004; Notara, Haddow et al. 2007). The EMEA accepts a number of clinical products that use animal products, so the use of an animal product containing culture system is not an absolute barrier to bring it to the clinic. However, the use of animal products bears the risk of disease transmission and therefore it cannot be seen as an ideal solution for the use in clinical applications.

We were able to show that mimicking an environment *in vitro* that is more similar to the stem cell “niche” *in vivo* improves the maintenance of conjunctival cells with progenitor cell characteristics (Schrader, Notara et al. 2010). Therefore, part of the future work will focus on further improvements on this culture system, but also on the exploration of new animal product free and feeder cell free culture systems with the aim to produce grafts with a high percentage of conjunctival progenitor cells under safe culture conditions for clinical use.

9.2. Evaluation of PC collagen gels as a substrate for the maintenance of conjunctival epithelial progenitor cells

The process for the controlled engineering of biomimetic scaffolds by rapid removal of fluid from hyperhydrated collagen gel constructs, using plastic compression (PC) was first reported by Brown et al. in 2005. In their study, PC fabrication produced dense, cellular, mechanically strong native collagen structures (Brown, Wiseman et al. 2005). As described in chapter 3.5 of this thesis, these PC collagen gels have shown to be suitable as a matrix for conjunctival epithelial cells.

The stroma of the conjunctiva also contains fibroblasts and tissue specific stromal fibroblasts are likely to play an important role in the regulation of progenitor epithelial cell differentiation both *in vivo* and *in vitro*, by providing growth factors and cytokines that could mediate complex paracrine interactions between epithelial cells and fibroblasts (Li and Tseng 1995; Spradling, Drummond-Barbosa et al. 2001). Therefore human conjunctival fibroblasts were seeded into the PC collagen gels at a ratio of approximately 1:3 to the epithelial cells, which is similar to the ratio used by Levis et al. (Levis, Brown et al. 2010) and also similar to the ratio used in the *in vitro* “niche” model described in chapter 3.3, which has shown to successfully support the maintenance of conjunctival epithelial progenitor cells (Schrader, Notara et al. 2010).

Future experiments are needed to evaluate whether PC collagen with or without fibroblast support can function as a niche system, which is capable to maintain a conjunctival epithelial progenitor cell population during *in vitro* culture but also *in vivo* after transplantation.

9.3. Mechanical evaluation of PC collagen gels as a conjunctival substitute

According to the break stress measurements described in chapter 3.5, the break stress values of the PC collagen gels were very similar to the human conjunctiva indicating similar strength of these tissues. However, these measurements were preliminary and more defined measurements using a more sophisticated mechanical testing device that can measure dynamic

stress-strain values have to follow. As the conjunctiva is exposed to permanent shear stress due to blinking and eye movements, an artificial substitute must also be evaluated by long-term strain tests and this has to be the aim of future experiments.

Finally the suitability of such a conjunctival construct has to be tested in an animal model to evaluate mechanical stability and the preservation of the epithelial cell population *in vivo*.

9.4. Steps towards a clinical application

As defined in the introduction an ideal conjunctival substitute should have a flexible matrix with a good long-term stability and elasticity.

The test of the biomechanical properties showed that no significant difference in break strength was found between human conjunctiva and PC collagen gels indicating similar biomechanical properties of the two substrates, however long-term stability and elasticity still needs to be evaluated.

Also an epithelial layer with a self-renewal potential on the surface of the matrix, which contains both epithelial as well as goblet cells was defined as an important property.

It was shown in this thesis that conjunctival epithelial cells with a self-renewal potential can be grown out of biopsies. These cells can be expanded *in vitro* and also differentiation into goblet cells was shown. Also these cells can be successfully transferred onto plastic compressed collagen gels. However future studies now have to confirm that after transfer to the gels cells with a

self-renewal potential are still preserved and it has to be evaluated if differentiation into goblet cells occurs on the gels.

Finally, a good biocompatibility and low immunogenicity is a crucial property needed for a construct aimed for transplantation.

These properties needed to be tested in an animal model. In ophthalmic research, rabbit models are most often used for experiments on the ocular surface, as their eyes and the ocular surface are relatively big, making examinations more easy compared to smaller animals like rats and mice. Also costs are relatively low and as it is a well known model, a lot of literature exists. In a first step only the matrix should be transplanted onto the conjunctival surface to evaluate whether it will be epithelialized by the hosts conjunctival epithelium and to assess whether it induces an immunogenic response in the host. In a second step the epithelialized construct should be transplanted to see whether the human conjunctival cells are able of self-renewal and if goblet cell differentiation occurs in the in vivo situation. However for these experiments the rabbits would need to be immunosuppressed, as epithelial cells tend to induce a strong immunogenic reaction by the hosts.

If the experiments, which are laid out above show promising results, it can be applied for ethic approval for a first in man transplantation which, if successful would pave the way for a phase I clinical trial.

In summary, the next steps towards a clinical application will be:

1. Completion of the in vitro work:

- Evaluation of long-term stability and elasticity of the PC gels**
- Test of self-renewal/goblet cell differentiation on the PC gels**

2. Test of biocompatibility and immunogenicity in a rabbit model

3. First in man transplantation

4. Phase I clinical trial

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Publications arising from this thesis

11. Publications arising from this thesis

11.1. Original articles

1. **Schrader S**, Notara M, Beaconsfield M, Tuft S, Geerling G, Daniels JT. Conjunctival epithelial cells maintain stem cell properties after long-term culture and cryopreservation. *Regen Med.* 2009 Sep;4(5):677-87.
2. **Schrader S**, Notara M, Tuft SJ, Beaconsfield M, Geerling G, Daniels JT. Simulation of an in vitro niche environment that preserves conjunctival progenitor cells. *Regen Med.* 2010 Nov;5(6):877-89.

11.2. Reviews

1. **Schrader S**, Notara M, Beaconsfield M, Tuft SJ, Daniels JT, Geerling G. Tissue engineering for conjunctival reconstruction: established methods and future outlooks. *Curr Eye Res.* 2009 Nov;34(11):913-24.